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Effect of Heterologous, Homologous, and Autologous Serums on Human Normal and Malignant Cells *In Vitro* [1,2,3]

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SUMMARY

Studies were conducted to evaluate the effect of autologous serum and Eagle's basal medium on primary short-term cultures of human malignant tissue. Such data would be useful in a long-range tissue-culture and clinical program in a comparison of the effects of certain chemotherapeutic agents on a tumor, both *in vivo* and *in vitro*, from the same patient. Forty-one tissue-culture series of "normal" and malignant tissues were included in a study comparing the effects of heterologous or horse serum, homologous or pooled human serum, and autologous serum, each combined with Eagle's basal medium, on the maintenance and growth of cells *in vitro*. Of 21 normal and malignant culture series, 19 exhibited better outgrowth characteristics in autologous serum in Eagle's medium as compared to cultures in horse serum with Eagle's medium. Autologous serum cultures in autologous serum plus Eagle's medium exhibited markedly less fibrinolysis. Sixteen series, which included normal and malignant tissues, generally exhibited better outgrowths in medium with autologous serum as compared to sister cultures in medium with pooled human serum. There was more than a twofold increase in the number of typical tumor cell types in medium with autologous serum. Individual tissue types displayed specific rates of migration and growth when they were cultured in the same percent of serum. However, cultures grew best in either Seitz-filtered or unfiltered autologous serum. Certain cell types, *viz.*, leiomyosarcoma and breast carcinoma, required as much as 50 percent autologous serum added to Eagle's medium for even limited growth *in vitro*. Of 52 tissue-culture drug series, 9 exhibited an increased sensitivity to actinomycin D, methotrexate (amethopterin), CB 1348 (chlorambucil), CB 3025 (phenylalanine mustard), and dihydro E-73 in horse or pooled human serum. One series, a melanoma, exhibited an increased sensitivity to dihydro E-73 in autologous serum.—J. Nat. Cancer Inst. 27: 1-15 1961.

THE PRESENT studies were undertaken to evaluate the effect of autologous serum on primary short-term cultures of human normal and malignant

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593953—61——2

tissue to simulate somewhat the environment *in vivo* from which the tissue was removed. Such studies were of value in a long-range tissue-culture and clinical program, currently in operation, to determine the correlation between the effects of promising cancer chemotherapeutic agents on a tumor *in vivo* and *in vitro* from the same patient.

Therefore, a pilot study was made to determine and compare the effects of heterologous or horse serum, homologous or pooled human serum, and autologous serum when each was combined with 80 percent Eagle's basal medium (EBM) on the maintenance and growth of malignant and normal cells *in vitro*. A few additional studies were also conducted to determine the responses of cells in the different serums plus EBM to cancer chemotherapeutic agents added directly to cultures.

Carrel and Ebeling (*1*) reported growth stimulation of fowl lymphocytes but inhibition of epithelial cells and fibroblasts with homologous serum in the medium tested. Lambert and Hanes (*2*) grew successfully several normal and malignant mammalian tissues in plasma from alien species. Later investigators have routinely and successfully employed heterologous media of several types, *e.g.*, plasma, embryo extract, and serum (*3*, *4*). Gey and Gey (*5*) described the cultivation of human normal and tumor cells in placental-cord (homologous) serum and bovine-embryo extract. Ivers and Pomerat (*6*) reported the successful use of autologous ascitic fluid for the cultivation of cells from effusions *in vitro*, and furthermore, recommended it as a component of media for homologous tissue cultures. Very little data exist in the literature, however, on the comparative effects of homologous and heterologous versus autologous serum on growth of sister cultures of normal and malignant tissue from the same individual.

MATERIALS AND METHODS

The horse serum was obtained from donor geldings maintained at the New York City Board of Health Farm, Otisville, New York. The serum was sterilized in our laboratory by Seitz filtration and stored at 4° C until needed. The pooled human serum was obtained from healthy donors at the Blood Transfusion Association, New York City, and sterilized by Seitz filtration. Some of this pooled serum was sent to our laboratory through the courtesy of Chas. Pfizer & Co., Inc., Brooklyn, New York. The human autologous serum was obtained from the patient at the time of biopsy. The sterile blood samples were allowed to clot at 4° C; the serum was then withdrawn and immediately incorporated into the nutrient medium for culture. A few samples of serum were sterilized through a hypodermic syringe fitted with a Seitz filter pad so that the differences could be evaluated in Seitz-filtered versus unfiltered serum. Adsorption of some serum nutrients to Seitz pads may have tended to yield less favorable results with filtered serums as compared to those with unfiltered serums.

EBM was obtained as a sterile, predissolved solution from Microbiological Associates, Bethesda, Maryland. As an assay procedure,

aliquots of the identical basal media were combined with the same batch of horse serum and used as nutrient medium for stock HeLa cultures that had been maintained for years in the laboratory. The cells so exposed were healthy as judged by microscopic examination. Aliquots of the same EBM were likewise combined with batches of the pooled human serum. All cells were healthy after exposure to these batches of media, which were chosen routinely for the maintenance of stock HeLa cultures.

Human malignant tissue was excised, or withdrawn, *e.g.*, by effusions, from patients with disseminated or advanced disease. Both primary and metastatic lesions from various anatomical sites were included. Normal tissues were obtained at surgery performed for (*a*) excision of a questionable enlarged lymph node, (*b*) herniorrhaphy, or (*c*) removal of adjacent "normal" tissue from patients with a diagnosed malignancy. The diagnoses of all specimens were confirmed by microscopic pathological examination. It is realized that the "normal" tissue of category (*c*) is classified only by morphologic criteria; true normalcy is difficult to prove.

The tissues were cut in 1 to 2 mm^3 fragments and placed on oblong coverslips, 11 × 22 mm, with 1 : 1 rooster plasma-chick-embryo-extract clot. Each culture series included 24 to 36 explants. Ascitic effusions were treated as cell suspensions on coverslips without plasma, as described earlier (*7*). Coverslip cultures were inserted in Porter flasks with 1 ml of nutrient solution composed of EBM (*8*) plus 20 percent of the serum to be evaluated. Neomycin, 150 γ/ml, was incorporated in all media. All nutrient solutions were adjusted to *p*H 7.2 with 0.1 N HCl prior to use. Microscopic observations of living cultures were made for 14 to 45 days, after which they were fixed in methyl alcohol and stained with Jenner-Giemsa reagent.

Tissue-culture series growing in the 3 serums were exposed in some experiments to one of the following test compounds currently under investigation as cancer chemotherapeutic agents: (*a*) actinomycin D,[6] 1×10^{-4} γ/ml; (*b*) methotrexate,[6] 0.1 mM; (*c*) CB 1348,[7] 0.5 mM; (*d*) thio-TEPA,[6] 0.1 mM; (*e*) CB 3025 or phenylalanine mustard,[7] 0.1 mM; and (*f*) dihydro E-73,[8] 1γ/ml. Only cultures with a minimal population of 1,000 cells were used for drug studies. The cell-population density was roughly determined by the insertion of a Howard grid in the eyepiece and the estimation of the number of cells in two 150× fields of each outgrowth.

Cultures were exposed for 96 hours to a test agent, when they were fixed and stained for cytological study. Those that exhibited cytotoxic changes were called sensitive; those with equivocal or no changes were called resistant.

The experiments in this study were divided into two groups: *Group I.*—Half of each individual culture series received EBM with horse serum and half, EBM with autologous serum. *Group II.*—Half of each series

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received EBM with pooled human serum and half, EBM with autologous serum.

RESULTS

Included in group I were 18 malignant and 3 normal tissues. The results observed in tissue culture for this group are summarized in table 1. Nineteen culture series, which included both normal and malignant tissues, exhibited better outgrowth characteristics in human autologous serum. Of this series, 10 exhibited moderate outgrowths in horse serum that were never as extensive as sister cultures in autologous serum. Cultures in this serum often displayed more mitoses, greater cell density and migration, and more typical tumor cell types than cultures in horse serum. An example of a different pattern of migration of cells grown in autologous serum is seen in a culture of a fibrosarcoma (fig. 1). A marked decrease in cell density near the explant and increased liquefaction

TABLE 1.—Effect of horse serum versus human autologous serum on malignant and normal cells *in vitro*

Number	Tissue type	Better serum*	Culture characteristics
1	Hodgkin's disease node	A	Mitoses in A only Pleomorphic cells in A only
2	Hodgkin's disease node	A	Mitoses slightly greater in A
3	Reticulum-cell sarcoma, lymph node	A	Mitoses in A only Fibrinolysis marked in Ho
4	Fibrosarcoma	A	Dense outgrowths in A only Fibrinolysis in Ho only
5	Melanoma, metastasis	A	Mitoses in A only Melanocytes marked in A
6	Melanoma, recurrent mass	A	Scanty outgrowth in Ho Mitoses in A only
7	Epidermoid carcinoma of mouth, primary mass	A	Wide epithelial sheets in both serums Mitoses in A only
8	Carcinoma of stomach, primary mass	A	Carcinoma cells in A only Scanty outgrowth in Ho
9	Carcinoma of colon, metastasis	Ho	Scanty outgrowth in both serums Ho better
10	Carcinoma of colon, metastasis	A	Mitoses in A only Carcinoma cells in both
11	Carcinoma of colon, recurrent mass	A	Carcinoma cells increased in A
12	Carcinoma of sigmoid, metastasis	A	Dense outgrowth in A only
13	Carcinoma of rectum, recurrent mass	A	Carcinoma cells in A only Stromal cells only in Ho
14	Carcinoma of kidney, recurrent mass	Ho	Increased cell density in Ho Mitoses in both
15	Carcinoma of breast, metastasis	A	Carcinoma cells increased in A
16	Carcinoma of breast, metastasis	A	Scanty outgrowth in A Rare cells in Ho
17	Carcinoma of thyroid, primary mass	A	Migration increased in A Carcinoma cells in both
18	Malignant mixed-cell tumor of parotid gland	A	Mitoses in A only
19	"Normal" lymph node (lymphadenopathy, etiology unknown)	A	Mitoses in A only Cell density 10× greater in A
20	"Normal" lymph node (TB patient, not involved)	A	Lymphocytes healthy in A only Mitoses in A only
21	"Normal" bile duct (adjacent to carcinoma of gallbladder)	A	Wide epithelial sheets in A Narrow in Ho

*A = autologous; Ho = horse.

of the plasma clot were noted in sister cultures grown in horse serum (fig. 2).

The cytology of the same cell types in the outgrowths of series grown in both serums was similar. The relative proportion of stromal cells to identifiable tumor cells was determined by microscopic examination of the outgrowths of 2 representative fields of each explant. A Howard grid was inserted in the eyepiece, and 2 fields examined at 150× and 430×. Cultures in horse serum had 0 to 2 tumor cells per 200× field. Cultures in autologous serum had 4 to 10 conspicuous tumor cells per 200× field. There were more pleomorphic cell types in Hodgkin's disease and reticulum-cell sarcoma tissues when grown in autologous serum. Fibrinolysis, however, was more pronounced in cultures with horse serum (figs. 3 and 4).

The second group of tissues was cultured in EBM plus pooled human serum and in EBM plus autologous serum. Included were 20 culture series, 18 malignant and 2 normal tissues (table 2). Sixteen tissues, including both normal and malignant, exhibited better outgrowth characteristics in autologous serum. They displayed more extensive migration (figs. 5 and 6), healthier lymphocytic elements, often increased cell density, a greater number of typical tumor cell types, and more mitoses than cultures in pooled serum. Differences in outgrowths between the two serums were not as marked in 3 of the 16 series in group II as differences observed between serums in most series of group I. One metastatic breast-carcinoma series grew better in pooled human serum. Fibrinolysis was increased in lymphoma and melanoma series in pooled human serum. While there were sometimes marked differences in the number of tumor cell types in the two serums, the appearance of those cells that migrated was similar.

A tissue from a recurrent rectal carcinoma (table 2, #10) was divided into 3 parts and prepared as sister cultures in EBM with horse, pooled, and autologous serums. Cultures in horse and pooled human serums developed a scanty outgrowth of stromal cells only (figs. 7 and 8). Cultures in autologous serum developed wide epithelial sheets (fig. 9) that became granular after 7 days.

Twenty percent autologous serum in EBM did not supply the nutritive requirements for migration *in vitro* and metabolism of tissues of a leiomyosarcoma and a primary breast carcinoma. When the serum concentration was increased to 50 percent, however, both culture series developed luxurious outgrowths. The breast carcinoma was also cultured in 50 percent pooled human serum, which also proved inadequate.

That the process of filtration of horse and pooled human serums through a Seitz filter pad may have resulted in the serums containing a toxic residue from the pad was considered as a possible reason for improved outgrowths in the unfiltered autologous serum. Therefore, 3 tissues were set up as replicate cultures and combined with nutrient medium composed of EBM plus 20 percent filtered autologous, horse, or pooled human serum. Cultures of a pleural effusion from a patient with breast carcinoma exhibited increased cell density and epithelial-cell types in autologous serum as

TABLE 2.—Effect of human pooled serum versus human autologous serum on malignant and normal cells

Number	Tissue type	Better serum*	Culture characteristics
1	Lymphosarcoma, lymph node	A	Increased mitoses in A Lymphocytes pyknotic in P
2	Lymphosarcoma, lymph node	A	Increased fibrinolysis in P Lymphocytes pyknotic in P
3	Hodgkin's disease, lymph node	A	Increased mitoses in A Pleomorphic cells in A only
4	Reticulum-cell sarcoma, lymph node	A	Increased mitoses in A only Migration minimal in P
5	Malignant lymphoma, lymph node, type undetermined	A	Cell density 4×greater in A Mitoses in A only
6	Malignant lymphoma, type undetermined	A = P	Rapid migration, and mitoses in both
7	Melanoma, recurrent mass	A	Increased mitoses in A Melanocytes in both
8	Epidermoid carcinoma of mouth, primary mass	A	Carcinoma cell sheets in A only
9	Epidermoid carcinoma of larynx, metastasis	A	Carcinoma cell sheets in A only No outgrowth in P
10	Carcinoma of rectum, recurrent mass	A	Carcinoma cell sheets in A only
11	Carcinoma of rectum, metastasis	A	More carcinoma cells in A Fibrinolysis in A only
12	Carcinoma of ovary, metastasis	A	Mitoses and carcinoma cells in A only
13	Carcinoma of breast, primary mass	A	Many mitoses in A No growth in P
14	Carcinoma of breast, metastasis	P	Giant stromal cells, few mitoses No growth in A
15	Malignant mixed-cell tumor of parotid gland	A	Outgrowth 3×greater in A Mitoses in A only
16	Glioblastoma	A	Dense outgrowth in A only
17	Reactive lymph node (lymphosarcoma patient)	A	Dense outgrowth in A No growth in P
18	Normal lymph node	A	Wide migration in A only Marked fibrinolysis in P
19	Leiomyosarcoma	50% A	No growth in 20% A or P
20	Carcinoma of breast, primary mass	50% A	Dense outgrowth, many mitoses in A

*P = pooled; A = autologous.

compared to scanty cell populations and fibroblast-like cells in horse and pooled human serums. Reticulum-cell sarcoma cultures exhibited a dense emigration of lymphocytic and stromal forms and frequent mitoses in autologous serum. Cultures in the other two exhibited scanty outgrowths with few mitoses. Cultures of the third tissue series, a lymphoma of undetermined type, also exhibited markedly better outgrowths in autologous serum as compared to the other two types of serums. Cultures in autologous serum developed clear, healthy lymphocytic elements and swirling stromal cells with many mitoses. Cultures in the other two serums exhibited scanty outgrowths of stromal cells plus rare pyknotic lymphocytes.

A few cultures from groups I and II were exposed as replicate cultures to one or several chemotherapeutic agents to determine whether there was an alteration of cell response in various serum types, and if so, in what manner. Twelve tissues of group I and 8 of group II were exposed to test

agents (table 3). Certain tissues, indicated by a single underline, cultured in EBM plus horse or pooled human serum, exhibited increased sensitivity to actinomycin D, methotrexate, CB 1348, CB 3025, and dihydro E-73. Increased sensitivity was manifested by an exaggeration of cytotoxicity. An example of such changes in a culture with 20 percent horse serum exposed to dihydro E-73 is seen in figure 11. The resistant culture with autologous serum is seen in figure 10. Sensitivity to CB 3025 was increased in autologous serum, indicated by the double underline, in one melanoma series.

TABLE 3.—Effects of chemotherapeutic agents in various serums*

Tissue type	Actinomycin D		Methotrexate		CB 1348		Thio-TEPA		CB 3025		Dihydro E-73	
I	Ho	A	Ho	A	Ho	A	Ho	A	Ho	A	Ho	A
1. Hodgkin's disease node	S	S							R	R		
2. Hodgkin's disease node					S	S	S	S	S	R†	S	R
3. Reticulum-cell sarcoma	S	S					S	S	S	S		
4. Fibrosarcoma			R	R			S	S				
5. Melanoma			R	R	S	S	S	S	R	R		
6. Epidermoid carcinoma of mouth	S	S							S>S		S	S
7. Carcinoma of colon	S	S										
8. Carcinoma of rectum			S	S					R	R		
9. Carcinoma of breast	S	S	S	S					R	R		
10. Carcinoma of thyroid	S>S				S	R	R	R				
11. Normal lymph node	S	S	R	R	S	S			R	R	S	R
12. Normal lymph node	S	S					S	S				
II	P	A	P	A	P	A	P	A	P	A	P	A
13. Lymphosarcoma	R	R	S	S			S	S				
14. Lymphosarcoma											S	S
15. Hodgkin's disease node					S	S	S	S	S	R	R	R
16. Reticulum-cell sarcoma			S>S		R	R					S	R
17. Melanoma							S	S	S<S‡			
18. Carcinoma of rectum											S	S
19. Normal lymph node											R	R
20. Carcinoma of breast in 50% serum	R	R			S	S	R	R				

*Ho = horse serum; S = cell injury; R = no cell injury; P = pooled human serum; A = autologous serum.
†Single underline indicates increased sensitivity by tissues cultured in EBM plus horse or pooled human serum.
‡Double underline indicates increased sensitivity by tissues cultured in autologous serum.

DISCUSSION

The data indicate that cells *in vitro*, when cultivated in a chemically defined medium supplemented with 20 percent serum, grew best in autologous serum whether of normal or malignant type. The experiments

of Fischer (*9*) indicated that chick fibroblasts thrived best in media supplemented with homologous proteins of high molecular weight, as compared to media supplemented with heterologous proteins of the same type. He postulated that in the homologous proteins the configuration of the polypeptide chains corresponds rather closely to that of the protein of which the cytoplasm is built, so that introduction of the new building units in the cell body is facilitated. This concept might well apply in the present experiments, since autologous serum probably possesses an even greater number of certain polypeptides than does homologous serum, identical with the configuration of certain nitrogenous building blocks needed by the cell.

The role of immunologic factors on cell activity awaits further studies comparing the effects of thermolabile versus thermostabile components of autologous, horse, and pooled human serums.

Autologous serum apparently elicited the emigration of certain normal and malignant epithelial-cell types while other serums were inadequate. Phillips and Andrews (*10*) noted that HeLa cells grown in medium containing human (homologous) serum respired and formed lactate at a greater rate than the same cells grown in horse serum. Cell types in homologous (pooled human) or heterologous (horse) serum were often similar. Differences between the cytology of basic cell types (as lymphocytes) in the three serums were not seen. Results in these data correlate with the observations of Olmstead and Rose (*11*) who determined the effects of various serums on neuroglia of the cat *in vitro*.

Epithelial cells often exhibited better migration in autologous serum than in horse or pooled human serum. This observation raises provocative questions regarding selective differences in cellular metabolism. The more demanding requirements of a tissue from leiomyosarcoma for 50 rather than 20 percent serum are of interest. Similarly, the need for 50 percent autologous serum by breast-carcinoma cells may indicate a requirement for an essential hormone present in the serum. The requirement for 50 rather than 20 percent pooled human serum for adequate growth of tissue from leiomyosarcoma and the ineffectiveness of even 50 percent pooled human serum for tissue outgrowth of breast carcinoma may represent the absence of an adequate amount of some essential hormone or agent in the pooled serum. Dilution of such a factor in the serum of one donor contributing to the total pool may occur.

Preferential and more efficient utilization of autologous serum by malignant or "normal" cells *in vitro* may be due to a more efficient utilization of autonomous agents in the serum. The more efficient metabolism may be due to 1) immunological properties of an essential component of the serum, or 2) adequate titers of a needed hormone present mainly in autologous serum.

Increased sensitivity to chemotherapeutic agents by cells in some horse or pooled human serum cultures was probably related to the health of the cells prior to treatment. Slightly less healthy cells were often more sensitive to drugs than healthier ones in autologous serum.

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PLATES

593953—61——3

Plate 1

Figure 1.—Fibrosarcoma culture after 17 days in EBM plus autologous serum. *Note* dense, even outgrowth. Jenner-Giemsa stain. × 25

Figure 2.—Fibrosarcoma culture after 17 days in EBM plus horse serum. *Note* fibrinolysis and moderately dense outgrowth. Jenner-Giemsa stain. × 25

Figure 3.—Living reticulum-cell-sarcoma explants on oblong coverslips with plasma clot after 48 hours in EBM plus autologous serum. × 2.5

Figure 4.—Living reticulum-cell-sarcoma explants on coverslips with plasma clot after 48 hours in EBM plus horse serum. × 2.5

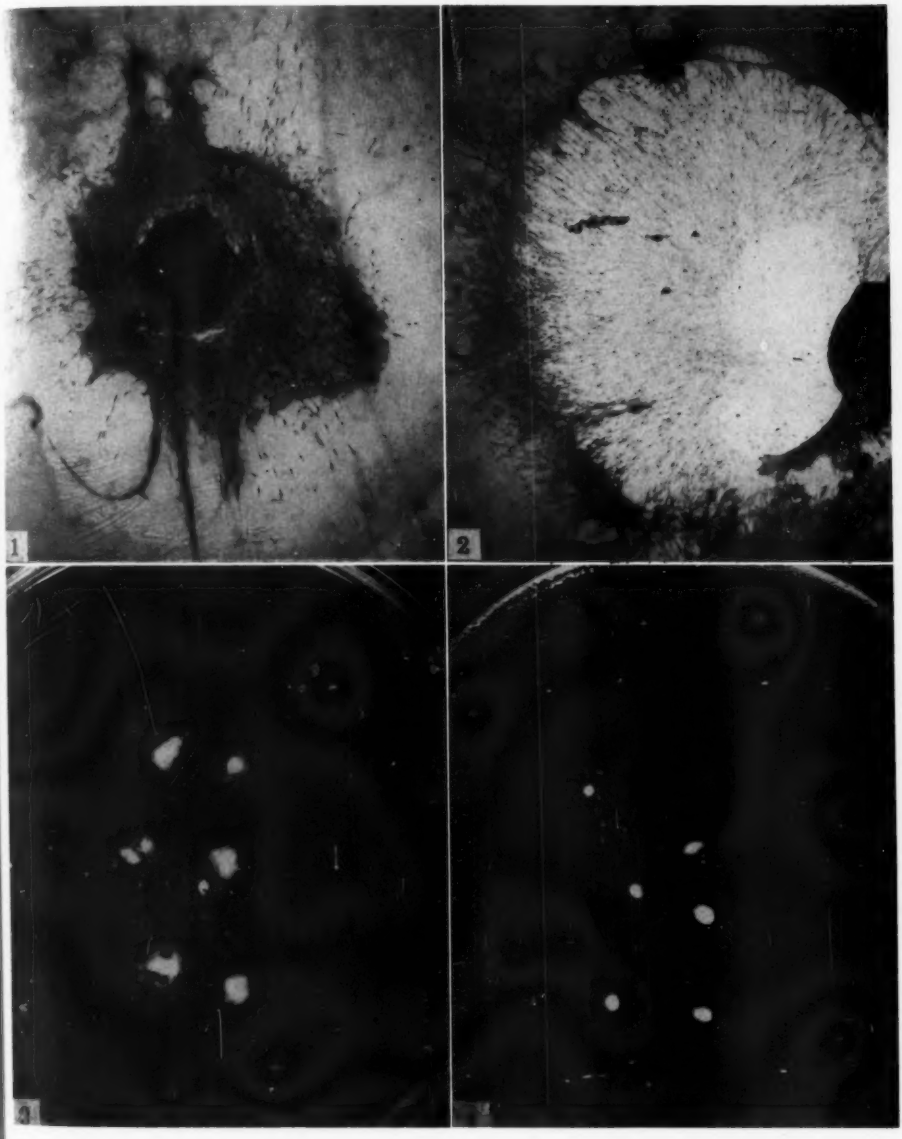
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Plate 2

Figure 5.—Melanoma culture after 13 days in EBM plus autologous serum. *Note* extensive migration. Jenner-Giemsa stain. × 75

Figure 6.—Melanoma culture after 13 days in EBM plus pooled human serum. *Note* fibrinolysis. Jenner-Giemsa stain. × 75

Figure 7.—Living rectal-carcinoma culture with scanty outgrowth after 8 days in EBM plus horse serum. × 110

Figure 8.—Living rectal-carcinoma culture with scanty outgrowth after 8 days in EBM plus pooled human serum. × 110

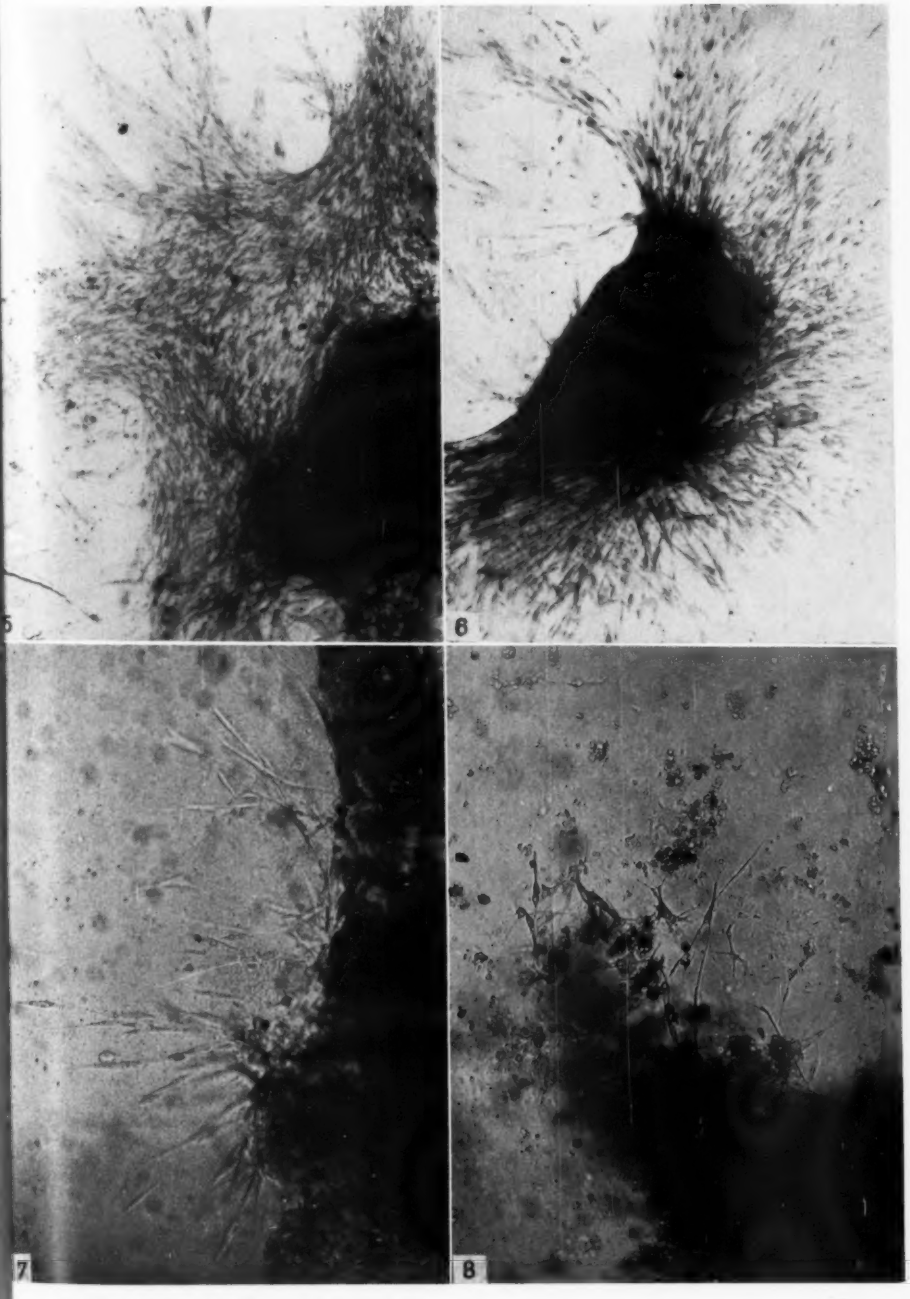
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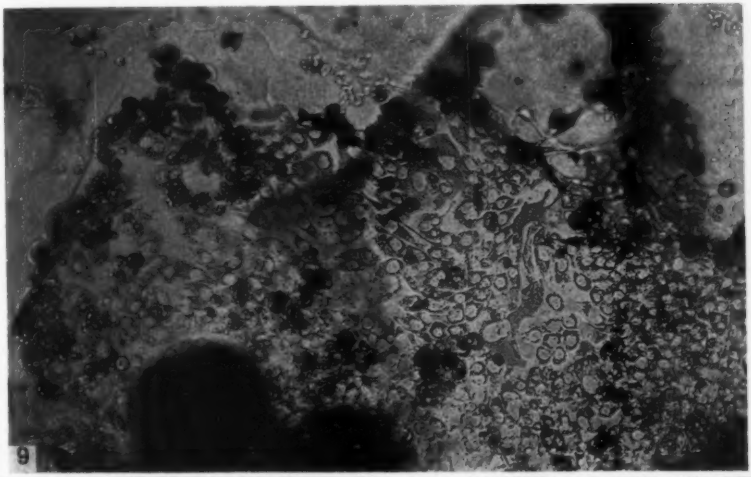
PLATE 3

FIGURE 9.—Living rectal-carcinoma culture with epithelial sheet formation after 8 days in EBM plus autologous serum. × 110

FIGURE 10.—Healthy cells in normal lymph-node culture after 13 days in EBM plus autologous serum and a 96-hour exposure to dihydro E-73. Jenner-Giemsa stain. × 280

FIGURE 11.—Damaged cells in normal lymph-node culture after 13 days in EBM plus horse serum and a 96-hour exposure to dihydro E-73. Jenner-Giemsa stain. × 280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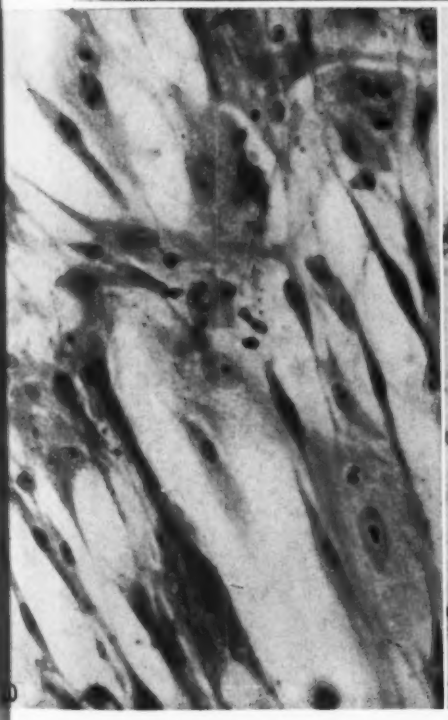

11

Effect of Heparin on Circulating Walker Carcinosarcoma 256 Cells[1,2]

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SUMMARY

Heparinization of the rat resulted in a significantly lower tumor-cell count in the systemic circulation after a single inoculation of 300,000 Walker carcinosarcoma 256 cells. The transplantability of these circulating cells was greatly decreased by the heparinization of the animal.—J. Nat. Cancer Inst. 27: 17-24, 1961.

THE DEMONSTRATION of cancer cells in the circulating blood after manipulation of a malignant tumor has stimulated interest in the factors affecting circulation and lodging of tumor cells (*1*). It has been shown that the alteration of the clotting mechanisms by heparin and human fibrinolysin affects the rate of pulmonary metastases after the intravenous inoculation of cancer cells (*2*, *3*).

Both heparin (*3*) and fibrinolysin (*4*) inhibited the formation of pulmonary metastases when the VX2 carcinoma was inoculated into rabbits (*3*, *5*). In studies of the transpulmonary passage of Walker carcinosarcoma 256 cells and mouse sarcoma, a decrease in pulmonary metastases was seen with heparin (*3*). Fibrinolysin also resulted in a diminution in pulmonary metastases with the Walker carcinosarcoma 256 in the rat (*2*) and in the number of pulmonary metastases after intravenous inoculation with Brown-Pearce carcinoma of the rabbit (*5*). A study of the incidence of hepatic metastases after the implantation of Walker carcinosarcoma 256 in the cecal pouch revealed that massage of the tumor resulted in an increase in hepatic metastases (*6*). The animals which received human fibrinolysin prior to the massage of the tumor had a much lower incidence of hepatic metastases than the controls (*7*).

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[6] The authors wish to thank Miss A. Layos for her technical assistance.

593953—61——4

When Walker carcinosarcoma 256 cells were inoculated intravenously, cells were found in the vena cava and aorta. The tumor cells disappeared rapidly from the systemic venous and arterial circulation. Human fibrinolysin given just prior to the carcinosarcoma cell suspension did not affect the rate of disappearance of the tumor cells, but the transplantability of the cells was greatly decreased (*1*). These effects of fibrinolytic enzyme on formation of metastases and transplantability of tumor cells have not been explained. The fibrinolytic enzyme prevents fibrin formation or dissolves fibrin, and it has been suggested that this prevents clumping or clustering of tumor cells necessary for fixation and growth *in vivo*.

To test this presumption, other means of altering the clotting mechanism are to be tested. The present experiment was set up to determine the effect of heparin on the fate and survival of Walker carcinosarcoma 256 cells.

MATERIALS AND METHODS

Adult Wistar female rats with an average weight of 200 g were used. The Walker carcinosarcoma 256 was carried in the ascitic form. Tumor cell suspensions from the ascitic fluid were made in saline and a dilution of 300,000 cells/ml was used. Aqueous heparin (Liquenamin, 1000 units/ml) was diluted to 50 units/ml in saline, and 1 ml of this dilution was given intravenously to each animal, about 250 units/kg. Clotting time was determined by the method of Lee and White (*8*). Direct counts of tumor cells in the blood were made by Seal's method (*9*). All inoculations were made into a surgically exposed femoral vein (*2*).

The control (primary recipient) animals were anesthetized with Nembutal 1:4, and 300,000 tumor cells were inoculated intravenously. Samples of blood were taken from the vena cava, aorta, and portal vein at intervals up to 3 hours. Direct counts of tumor cells were made. Five ml of blood was obtained from each primary recipient rat and a 1 ml aliquot of blood was inoculated subcutaneously into groups of 5 young rats (secondary recipients). The primary recipient and donor rat was then killed.

In the treated (primary recipient) group, 50 units of heparin were inoculated intravenously and 10 minutes later a tumor-cell suspension of 300,000 Walker carcinosarcoma 256 cells was also given intravenously. Samples of blood were taken, cell counts made, and aliquots of blood inoculated subcutaneously into weanling animals (secondary recipients), as with the controls.

A total of 1,592 animals was used in this experiment:

(*a*) Two hundred forty-one adult rats weighing 180 to 200 g, as primary recipients for each bioassay experiment, 5 rats for each point as shown in text-figures 1, 3, and 5.

(*b*) Fifty adult rats of 180 to 200 g in weight, for quantitative cell count, 1 animal for each point as shown in text-figures 1, 3, and 5.

(*c*) Twelve hundred weanling rats of 30 to 90 g in weight, as secondary recipients for subcutaneous inoculation of tumor cells, 25 animals for each point as shown in text-figures 2, 4, and 6.

(*d*) Eighty-one rats weighing 100 to 150 g, for the clotting-time study (table 1). This experiment was repeated 3 times.

(*e*) Twenty rats weighing 100 to 150 g, for the study of the effect of heparin on tumor cells *in vitro*. Cells (300,000) were suspended in 1 ml of heparin solution (50 mg) and allowed to stand at 60° C.

One ml of the mixture of tumor cells and heparin was inoculated subcutaneously into 5 animals at intervals of 5, 10, 15, and 30 minutes.

RESULTS

The effect of 50 units of heparin on the clotting time of a 200 g rat is shown in table 1. The clotting time is prolonged significantly in 10 minutes and remains prolonged for at least 2 hours after the injection. The effect is similar when the heparin is given alone or with the tumor suspension. The tumor suspension itself shortens the clotting time.

TABLE 1.—Clotting time of blood of rats inoculated intravenously with heparin and Walker carcinosarcoma 256 cells*

Control	50 USP heparin alone	Tumor cells 300,000 alone	Heparin 50 USP and tumor cells mixed
	2 Min	2 Min, 10 sec	1 Min, 30 sec
10 Min	11 Hr, 5 min	3 Min, 50 sec	9 Hrs
15 Min	5 Hr, 10 min	1 Min, 40 sec	7 Hrs, 30 min
30 Min	3 Hr, 20 min	1 Min, 50 sec	5 Hrs, 15 min
45 Min	3 Hr, 5 min	1 Min, 30 sec	6 Hrs
1 Hr	4 Hr, 10 min	1 Min	4 Hrs, 20 min
2 Hrs	2 Hr, 15 min	1 Min, 30 sec	3 Hrs
3 Hrs	2 Hr, 10 min	1 Min, 50 sec	1 Hr, 45 min
4 Hrs	5 Min	2 Min	2 Min

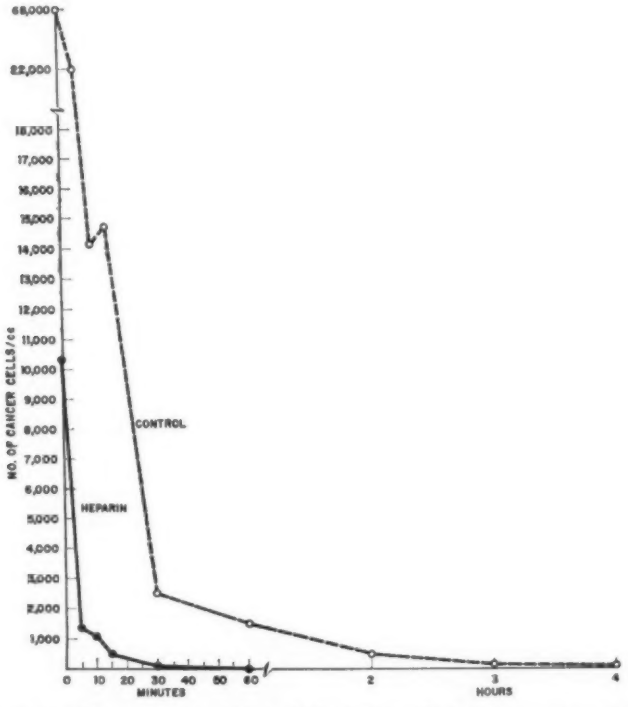
*According to the method of Lee and White (5).

Subcutaneous inoculation of tumor cells incubated with heparin for 5, 10, 15, and 30 minutes resulted in tumor growth in 100 percent of the animals. The concentration of heparin, 50 mg/ml, was much greater than that obtained in the blood of the animals which received only 50 mg/animal (approximately 20 ml blood volume).

Femoral-Vein Injection—Blood Drawn From Inferior Vena Cava

The tumor-cell counts in the vena cava blood, after the intravenous inoculation of 300,000 Walker carcinosarcoma 256 cells into the femoral vein of control and heparinized rats, are shown in text-figure 1. The count was much higher in the control group throughout the 4-hour observation period. There was a rapid fall in the tumor-cell count in the first 30 minutes, and then a gradual decrease in the number of cells, until none

were found at 4 hours. At 5 minutes there were 22,000 cells/mm³ in the controls and 1,200 cells/mm³ in the heparinized animal. At 15 minutes the control had 14,600 cells/mm³, while the treated animal had less than 400 cells/mm³. After 1 hour no tumor cells were found in the vena cava blood of the heparinized animals.

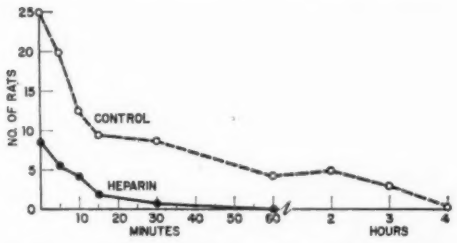


TEXT-FIGURE 1.—Tumor-cell count/ml of blood from the inferior vena cava in rats inoculated with 300,000 Walker carcinosarcoma 256 cells via the femoral vein. Time after inoculation is given. In this and other figures, *broken* line represents the control group and *solid* line the heparinized animals.

Subcutaneous inoculation of the blood into donor animals resulted in many more takes in the controls than in the heparin-treated group (text-fig. 2). With blood drawn at 1 minute, there was growth in 100 percent of the animals inoculated with blood from the controls and in 64 percent of the animals inoculated with blood from the heparinized animals. The number and percent of growths in the heparinized animals decreased rapidly—to 0 at 1 hour. The controls continued to have takes at 2 and 3 hours. The recipient inoculated rats were observed for 9 weeks (text-fig. 2).

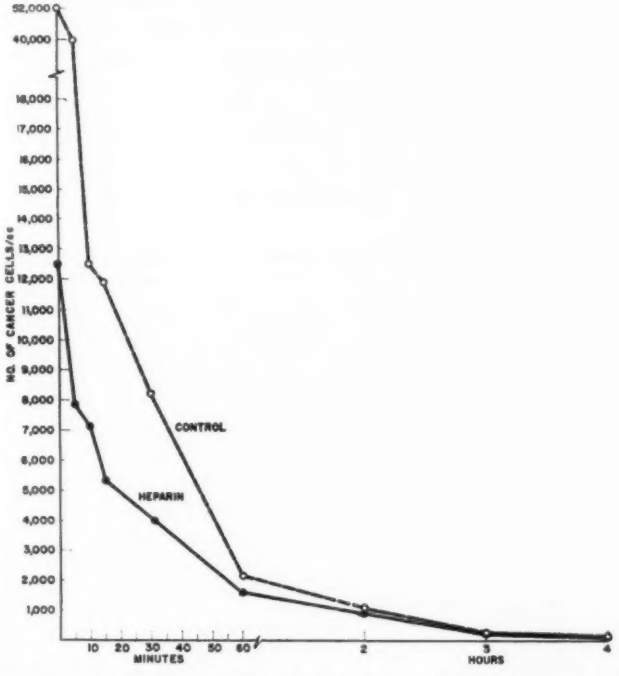
Jugular-Vein Injection—Blood Drawn From Inferior Vena Cava

When the tumor cells were inoculated into the jugular vein and blood samples were taken from the inferior vena cava, tumor-cell counts similar



TEXT-FIGURE 2.—Tumor growth in animals inoculated subcutaneously with blood from the inferior vena cava of donor rats. Total number of rats each time was 25.

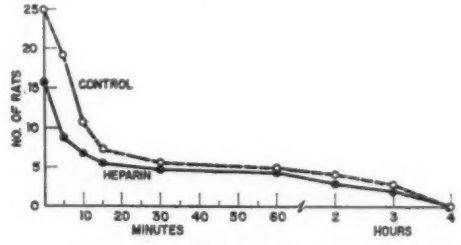
to the group inoculated via the femoral vein were obtained (text-fig. 3). In the control group of rats, there was a slow decrease in the number of circulating cancer cells from 52,000 to 2,200 at 1 hour. In the heparinized rat, the number of cells was only 12,400 at 1 minute and was much lower throughout the period of observation.



TEXT-FIGURE 3.—Tumor-cell count/ml of blood from the inferior vena cava in rats inoculated via the jugular vein.

With the inoculation of blood from the vena cava into the donor animals, the results were even more striking (text-fig. 4). The number of takes at 1 minute was the same, 100 and 64 percent as in the previous group. The

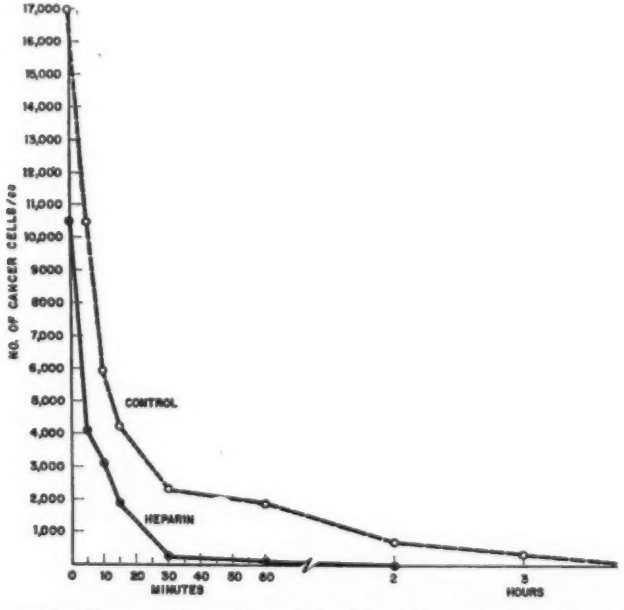
decrease was not so rapid in this group, though the blood from the heparinized animals always gave less takes, until 4 hours when no growth occurred with either control or heparinized blood.



TEXT-FIGURE 4.—Tumor growth in animals inoculated subcutaneously with blood from the inferior vena cava of the animals shown in text-figure 3.

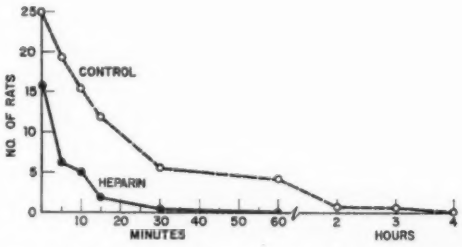
Aortic Blood

When direct tumor-cell counts of the aortic blood were made after inoculation of 300,000 Walker carcinosarcoma 256 cells into the femoral vein, the same relationship with significantly less cells in the heparinized than the control animals was noted (text-fig. 5). In the controls the counts in aortic blood were much lower than in the vena cava blood. In the heparinized animals aortic and vena cava blood contained about the same number of cells (text-figs. 1 and 5).



TEXT-FIGURE 5.—Tumor-cell count/ml of blood from the aorta in rats inoculated via the femoral vein.

Subcutaneous inoculation of aortic blood from the controls into weanling rats again gave a higher rate of growth than that from the heparinized animals (text-fig. 6). Again the takes were 100 percent at 1 minute in the control group. In the rats treated with heparin, takes occurred in 30 percent at 1 minute and rapidly decreased to 0 at 60 minutes as in the first vena cava group. The curve is similar for these two groups of animals (text-figs. 2 and 6).



TEXT-FIGURE 6.—The rate of tumor growth in recipient animals inoculated subcutaneously with blood from the aorta.

We failed to demonstrate tumor cells in the blood from the portal vein in controls or heparinized animals. Inoculation of this portal-vein blood failed to result in any tumor formation in the recipient (weanling) rats.

DISCUSSION

The present data support the contention that alterations in coagulation mechanisms and the prevention of fibrin formation or coagulation seem to bring about a more rapid disappearance of cancer cells from the blood stream and apparently hinder their successful transplantation. The difference between the control and treated animals in direct tumor-cell counts is striking and uniform. That the jugular-vein route gives results similar to the femoral vein inoculation indicates very little stasis of the tumor cells prior to circulation through the lungs. The transpulmonary passage of tumor cells is clearly shown by their presence in the aortic blood. The marked diminution in the number of tumor cells and the decreased ability to transplant them from both the venous and aortic blood when the animals were heparinized are very striking and significant.

The absence of tumor cells in the portal-vein blood confirms our findings in the investigations with fibrinolysin (*1*). This must be of importance in our understanding of the mechanism of tumor metastases. Are the cells held in the splanchnic area and fail to grow, or are they filtered out and returned to the circulation by the lymphatics? Additional investigations to determine the presence of tumor cells in the thoracic duct lymph or in the splanchnic area are indicated.

Further experience with other easily transplantable tumors will be necessary before a clinical application can be considered.

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Fatty Acid Composition of Adipose Tissue in Patients With Carcinoma of the Breast [1, 2]

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SUMMARY

The percent composition of the fatty acids in breast and subcutaneous adipose tissue in 11 patients with breast carcinoma was compared with that of the fatty acids in subcutaneous adipose tissue in 10 random controls. No significant difference in the percent composition of the major fatty acids with a carbon chain length of C12 to C18 was demonstrated by analysis with the use of gas-liquid-phase chromatography.—J. Nat. Cancer Inst. 27: 25–28, 1961.

THE FATTY acid composition of human adipose tissue has been determined with respect to age, anatomical site, diet, and a number of clinical states (*1*). There have been no reports on the fatty acid composition of adipose tissue in patients with cancer. The possiblility that there might be an alteration in the fatty acid composition of adipose tissue in patients with breast carcinoma prompted the present study.

METHODS

Small, 100 to 500 mg, samples of adipose tissue were obtained from two sites in the resected specimens of 11 patients undergoing radical mastectomy for breast carcinoma at the Medical College of Virginia Hospitals. Samples were taken from a subcutaneous site approximately 3 cm from the nipple and a site high in the axillary area. In 10 random control patients, whose conditions are listed in table 1, samples were taken from a subcutaneous site at the point of surgical incision. Pathological diagnosis in the patients studied was confirmed by the Department of Surgical Pathology.

The lipides present in the samples were extracted by the method of Folch *et al.* (*2*). Aliquots of the extract were transesterified by the

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[3] The author is indebted to Dr. Allan L. Forbes, Veterans Hospital, Richmond, Va., who made available an established gas chromatograph and column designed for fatty acid analysis, and Elizabeth Cauthorne, M. T., Veterans Hospital, for technical assistance.

TABLE 1.—Pathological diagnosis in cases reported in this study

Patients: age, race, sex	Pathological diagnosis, breast carcinoma	Patients: age, race, sex	Pathological diagnosis, random controls
41 W F	Scirrhous	40 C F	Chronic cystic mastitis
62 W F	Scirrhous	48 W F	Chronic cystic mastitis
46 W F	Scirrhous	50 W F	Gallbladder
66 C F	Scirrhous	35 W F	Fallopian tubes, appendix
51 W F	Scirrhous	66 W F	(Traumatic hip fracture)
52 C F	Scirrhous	61 W M	Acute appendicitis
74 W F	Intraductal	82 W M	Hernial sac, inguinal
79 W F	Intraductal	33 W F	Chronic cholecystitis, cholelithiasis
57 W F	Intraductal, with invasion	50 W F	Papillomatosis, mammary gland
74 C F	Lobular, infiltrating, metastatic to axilla	21 W F	Adenofibroma, mammary gland
67 W F	Inflammatory, metastatic to axilla		

method of Stoffel *et al.* (*3*), with the substitution of 2 percent H_2SO_4 CH_3OH for 5 percent HCl CH_3OH. The methyl esters of the fatty acids in each sample were taken to dryness with a nitrogen stream and made up to approximately a 5 percent solution in *n*-hexane. Each sample was then analyzed by gas-liquid-phase chromatography (GLC).

The gas-liquid-phase chromatograph used was a Pye-Argon instrument with ionization detector, adapted with a preheater and a serum cap for injection of the sample by syringe. The carrier gas was argon, and the column 4 feet long with an inside diameter of 4 mm packed with ethylene glycol succinate polyester as the stationary phase.

Percentage composition of the fatty acids present was calculated for the major fractions by triangulation. Identity of the fractions was determined by a comparison of retention times (volumes) with a known standard containing a mixture of the methyl esters of palmitic, 16:0; stearic, 18:0; oleic, 18:1; linoleic, 18:2; and linolenic acid, 18:3. We established identity by checking a plot of carbon chain length versus the log of retention time, using stearic acid as 1:00. The close correlation with published data from other laboratories is illustrated in table 2. There were usually 20 discernible fractions on the obtained graph, but only 9 major fractions were triangulated in the determination of the percent composition.

RESULTS

The data obtained by GLC analysis (table 2) give the percent composition of the methyl esters of the fatty acids in human adipose tissue in 3 groups. Group I consists of samples obtained from the breast of 11 patients with breast carcinoma, Group II, samples obtained from the axillary area of the resected surgical specimens in the same patients as Group I. Group III contains 10 random control patients.

TABLE 2.—Comparison of fatty acid composition of adipose tissue in axillary, mammary, and control groups, with two published reports

Fatty acid*	Group I (mammary) Percent	SD	Group II (axillary) Percent	SD	Group III (controls) Percent	SD	Hirsch *et al.* (*1*): GLC—12 cases Percent	SD	Cramer *et al.* (*4*): classic methods, mean of 5 cases
12:0	0.5	0.2	0.6	0.1	0.6	0.3	0.7	0.1	0.5
14:0	2.8	0.9	2.8	0.6	3.0	0.7	3.3	0.1	3.5
14:1	0.9	0.5†	0.8	0.4†	0.8	0.4†	0.6	0.05	0.4
16:0	21.4	1.7	21.4	1.5	20.8	1.5	19.5	2.1	25.0
16:1	5.9	1.4	5.8	1.3	6.1	1.7	6.9	0.1	6.4
18:0	7.7	2.5	7.8	2.2	8.3	1.3	4.2	1.1	7.0
18:1	44.6	2.1	45.2	2.7	44.0	2.5	41.2	4.4	45.9
							5.1‡	1.0	
18:2	14.0	2.3	13.5	2.2	14.5	2.5	11.4	1.4	9.6
18:3	2.2	0.7	2.1	0.6	1.7	0.8	0.4	0.1	
20:4							0.2	0.1	0.7
Other 20 carbon acids									1.2

*Listed as carbon chain length with number of double bonds after colon.
†Fraction not definitely identified as 14:1.
‡18:1 Isomers.

DISCUSSION

The data demonstrate that the fatty acid composition of adipose tissue in the breast is the same as that in the axillary area for the major fatty acids with a carbon chain length of C12 to C18. This supports the report of Hirsch *et al.* (*1*) on the homogeneous character of subcutaneous adipose tissue in man. The data demonstrate that there are no significant differences in the major fatty acids in human adipose tissue in 11 patients with breast carcinoma as compared with 10 random controls.

The significant difference in the percent stearic acid, 18:0, present, in comparison of Hirsch's data with our own, may represent variation in the population sample, possibly of dietary origin.

The fatty acid composition of adipose tissue is undoubtedly influenced by many metabolic factors, and one might expect the quantity of dietary fat, caloric effects, and hormonal factors to be important determinants (*1*, *5*, *6*). All these factors have been implicated in the experimental production of breast carcinoma in rodents (*7*).

The present study was based upon the hypothesis that the fatty acid composition of adipose tissue might reflect some difference in dietary habits or host lipide metabolism in patients who developed breast carcinoma as compared with a control group. On the basis of the data obtained, the hypothesis no longer appears tenable. It is, of course, possible that unidentified trace components and longer chain length fatty acids may differ in the groups studied. It was not possible to compare these small fractions in a quantitative manner.

It has been suggested that the fatty acid composition of adipose tissue may reflect that of the diet integrated over a long period of time (*1*, *5*). If this proves to be true, the possibility of a difference existing in the quality of fat in the diets of the study and control groups seems unlikely.

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A Hepatitis Virus Complicating Studies With Mouse Leukemia [1]

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SUMMARY

Hepatitis was frequently observed in strain BALB/c mice during the course of studies with the Moloney murine leukemia virus. A virus isolated from mice with hepatic disease was propagated and quantitatively assayed by methods *in vitro*. The properties of this agent, designated MHV-A59, were detailed, and a relationship to one member of the established mouse "hepatoencephalitis" group of viruses was ascertained.—J. Nat. Cancer Inst. 27: 29–51, 1961.

INTERFERENCE BY contaminating viruses that produce inapparent infection *in vitro* or *in vivo* poses a major problem for the acquisition and interpretation of experimental results in viral oncology. An apparently related group of agents associated with hepatitis in mice provides an example. Reports relative to this disease, originating in laboratories scattered around the world, are indicative of the wide dissemination of such agents in laboratory mouse populations (*1–8*). In some cases, overt disease after experimental treatment of animals has been the only evidence of latent infection in a colony, though isolations from untreated mice have been made (*3*, *4*, *7*, *9*). Virus activation has been observed after infection with the blood parasite *Eperythrozoon coccoides* (*5*, *10*), and after inoculation of mice with tissue or tissue extracts (*2*). Activation of latent infections has been particularly troublesome in animals used for studies on the induction of tumors by cell-free filtrates or for the transplantation of neoplasms (*6*, *8*, *11–13*).

The occurrence of hepatitis as a complicating factor in leukemia research has been reported with sufficient frequency to justify special attention. In the serial transplantation of lymphoid neoplasms in Princeton (Pr) and BALB/c mice, Nelson observed that loss of tumor cells was related to the activation of latent viral-hepatitis infections in his stock mice (*8*, *11–13*). Similarly, a passage of lymphocytic leukemia conducted by

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

means of cell-free filtrate in C3H mice by Kassel and Rottino was accompanied by the occurrence of acute hepatitis in the inoculated animals (*6*). The results of Braunsteiner and Friend (*1*) indicate that tumor cells may themselves carry virus. The presence of a hepatitis agent contaminating their line I leukemia could be detected by the treatment of mice bearing the transplanted neoplasm, with urethan or methylformamide.

During studies in our laboratory with the Moloney murine leukemia virus (*14*, *15*), 2 viruses that produce hepatic disease and death in suckling mice were isolated from the same BALB/c strain stock of mice. One of these viruses was obtained from livers of suckling mice dying with acute hepatitis 3 weeks after neonatal injection of a cell-free extract prepared from leukemic mice. The other virus was isolated from mice in which the leukemia agent was serially transmitted by means of minced, pooled liver, thymus, spleen, and lymph nodes.

Preliminary observations indicated that the latter agent was resistant to the action of anesthetic ether. In addition, mice that recovered from the disease showed resistance to subsequent infection with the agent. Previously described members of the mouse hepatitis group of viruses evoked a poor antigenic response (*16*). At least 2 of these viruses, the MHV-1 virus of Gledhill and Andrewes (*4*) and the JHM virus of Cheever and coworkers (*3*), were inactivated by ether. It appeared that a different agent was responsible for the hepatic disease in our animals. The behavior of this virus *in vivo* and *in vitro* and its relationship to previously described mouse hepatitis viruses are presented herein.

MATERIALS AND METHODS

Mice.—Mice from the National Institutes of Health Animal Production Section were used. They were housed in stainless-steel animal cages, one litter per cage, and were given Purina laboratory chow and water *ad libitum*.

Virus.—Beginning with the tissues from a BALB/c mouse bearing the sixth-transplant passage of cell-free, extract-induced leukemia (*14*) obtained from Dr. John B. Moloney, we made 30 serial intraperitoneal passages of minced mixtures of liver, spleen, thymus, and lymph nodes at 4- to 5-day intervals in 4- to 6-week-old mice. The animals in the 30th passage appeared ill. Their coats were rough and they huddled together in hunched positions. Two further passages of the pooled tissues were made. The livers in animals of these passages were pale and studded with focal white lesions. A 10 percent saline homogenate was prepared from the livers and spleens of animals representing the 32d passage. Filtration through a bacteria-tight 03 Selas filter provided a bacteriologically sterile extract that produced extensive necrosis of the liver after intraperitoneal injection into young mice. This constituted the initial working pool of the mouse hepatitis agent. The isolate was designated MHV-A59.

Serum.—All serums used in culture media were heated at 56° C for 30 minutes before use. Mouse serums tested for virus-neutralizing activity were heated at 60° C for 30 minutes.

Mouse liver line (ML) cultures.—Cultures of Earle's cloned mouse liver line, NCTC 1469, received through the courtesy of Dr. Virginia Evans of the National Cancer Institute, were used to originate a stock line providing cells for studies *in vitro*. Culture flasks or tubes were gassed with 5 percent CO_2 in O_2 prior to the addition of ML cells. Stock cells were cultured in flasks with Earle's NCTC 109 medium (*17*) containing 10 percent horse serum without antibiotics. For routine use, we prepared tube cultures in 16 × 120 mm tubes by seeding each with 1 ml of medium containing 5×10^5 cells. Antibiotics, usually 100 units of penicillin and 100 μg of streptomycin per ml of medium, were added. Nutrient fluids in all cultures were replaced 3 times each week.

Mouse spleen cultures.—Spleens removed from 5 to 8 weanling mice were pooled and minced with scissors. The minced tissue was washed 3 times with Hanks' saline by decantation of supernatant fluids after heavier tissue had settled. The washed tissue was incubated for 30 minutes at 37° C in 20 ml of Hanks' saline, *p*H 7.4, containing 0.25 percent Difco 1:250 trypsin. Vigorous pipetting at intervals during incubation dispersed the cells, which were sedimented by low-speed centrifugation. One wash with growth medium was made. Packed cells were suspended in 150 parts of medium consisting of 3 parts human serum and 7 parts of yeastolate medium without added sodium bicarbonate (*18*). One hundred units of penicillin and 100 μg of streptomycin per ml of medium were added. The cell suspension was dispensed into 16 × 120 mm tubes in 1 ml amounts. The tubes were not gassed with CO_2. After 3 to 4 days of incubation in slanted racks at 37° C, the fluids were replaced with 1.5 ml of medium. If a heavy cell population created conditions that were too acid, 1.4 percent sodium-bicarbonate solution was added to the medium as required. Routine fluid changes were made 3 times each week.

Titration of virus.—Virus titrations *in vivo* were usually made by intraperitoneal injection of 0.1 ml of virus dilution into 2- to 3-day-old suckling mice. Survivors were killed 1 week later and examined for liver lesions. Most animals died 3 to 6 days after inoculation. Deaths during the first 2 days were considered nonspecific and were not included in the data. Titers were calculated by the method of Reed and Muench (*19*). Titrations *in vitro* were made in culture tubes, seeded with ML cells 24 hours before, by the addition of 0.1 ml of virus dilution per tube. Results were obtained within 4 days of infection on the basis of destruction of cells by virus.

Virus neutralization.—The virus-neutralization titer of mouse serums was determined *in vitro* by a method similar to that described by Rowe *et al.* (*20*). Phosphate-buffered saline, *p*H 7.25, was used to dilute serums and virus. One part of a dilution containing 100 tissue-culture infectious doses (TCID50) of virus was mixed with 1 part of a serum dilution and

incubated at room temperature (24° C) for 2 hours. Serums previously heated at 60° C for 30 minutes were diluted in fourfold steps. After incubation, 8 parts of 109 medium containing 10 percent horse serum and antibiotics were added, and 0.8 ml of the mixture was used to replace the medium in each of 2 ML culture tubes. Virus control tubes received diluent in place of the diluted serum; serum control tubes received only diluent in place of the diluted virus. The tubes were incubated without further fluid change. Cytopathic changes were recorded daily over a 4-day period. Titers were read as the reciprocal of that serum dilution which, when mixed with 100 TCID50 of virus, prevented cytopathic changes in both tubes inoculated.

We demonstrated virus neutralization *in vivo* by mixing equal parts of a serum dilution with each of 2 dilutions of virus. After 2 hours of incubation at room temperature, 0.01 ml volumes of the mixture were injected intracerebrally into 1- to 4-day-old suckling mice of the BALB/c strain. A series of virus dilutions mixed with phosphate-buffered-saline diluent in place of diluted serum provided the reference control. Deaths occurring between the 2d and 7th days were considered specific. Survivors were killed 7 days after inoculation. All animals not cannibalized were examined for liver lesions. An estimate of the neutralizing activity of a serum was obtained by comparison with the animal response to the control inoculums.

OBSERVATIONS

Characteristics of MHV-A59 Infection *In Vivo*

Pathology.—Disease in weanling BALB/c mice 24 to 48 hours after intraperitoneal injection of A59 virus was indicated by roughened coats and a hunched position. Focal lesions, visible on the surface of the liver, were present 24 hours after virus inoculation. About one half of the animals inoculated died with extensive hepatic necrosis within a week. The gross pathological changes were similar to those described by Nelson (*8*). Histologic studies were carried out on groups of mice killed at intervals of 24 hours between the time of inoculation and the time when deaths usually began, at 4 days post inoculation. Sections were stained with hematoxylin and eosin or Giemsa. Sections of the liver, spleen, pancreas, heart, lungs, thymus, kidneys, central nervous system, gut, and mesenteric lymph nodes were examined. The liver, spleen, and lymphoid tissue of the gastrointestinal tract were altered as early as 24 hours after inoculation. Other organs appeared unaffected throughout the 4-day period of observation, except for a nonspecific inflammatory reaction of the peritoneal surfaces of the pancreas, liver, and spleen in several mice. This reaction was not extensive, and consisted primarily of mononuclear infiltrates. Giant cells were not seen.

In the liver, the formation of minute circumscribed foci of polymorphonuclear leukocytes preceded damage to hepatic cells. At 24 hours the

lesions, sparsely scattered throughout the parenchyma, occurred most commonly beneath the capsule or in close apposition to large branches of the portal vein (fig. 1). By 48 hours these lesions had become more numerous and in most instances leukocytes were no longer present. The foci consisted of fragmented nuclear debris and roughly demarcated areas of hepatic cells that had undergone coagulative necrosis (fig. 2). The affected cells had pyknotic nuclei, and a dense eosinophilic coagulum replaced the usual glycogen-laden granular cytoplasm. At later periods the process of coagulative necrosis appeared to extend from cell to cell until individual foci became confluent. At 96 hours, only those hepatic cells adjacent to central veins appeared undamaged (fig. 3). Little or no inflammatory cell infiltration and only moderate congestion of sinusoids followed the initial focal inflammatory reaction. Evidence of hepatic-cell regeneration was minimal during the first 96 hours. The lesion in the liver at the time of death generally consisted of a diffuse, bland necrosis of liver cells with dissociation of the hepatic plates.

In the spleen and lymph nodes small, isolated foci of necrosis were found, particularly during the first 48 hours. Such areas were heavily infiltrated with neutrophils and in some instances contained multinucleated giant cells (figs. 4 and 5). The giant cells appeared to be conglomerates of reticular cells unrelated to vascular endothelium. In the spleen, lesions of this type were characteristically distributed in the perifollicular regions and at the extreme periphery of the follicles. Congestion was much more intense in the lymphoid organs than in the liver, and hyperplasia of reticular cells was noted in the splenic red pulp of some, but not all, animals. No other cytological alterations could be identified that would distinguish this lesion from those caused by many other hepatotoxic agents. Microorganisms or inclusion bodies were not found in any organ examined.

Biological response to the virus.—Mice could be infected by intraperitoneal, intracerebral, oral, or subcutaneous inoculation. The intraperitoneal route was most satisfactory and was used unless otherwise specified. Eight serial virus passages were made in 6- to 8-week-old BALB/c mice by means of extracts of diseased livers. Titrations made in suckling mice indicated about 6.0 log LD50 of virus was extracted from a gram of liver tissue. Brain-to-brain passage was also made in 4-week-old BALB/c mice. In the course of 10 such passages no paralysis was noted, nor were convulsions observed when animals were suspended by their tails and twirled.

Although the majority of suckling strain BALB/c mice died within a week after virus inoculation, some litters were partially resistant and others were completely resistant. Rough estimates of virus titer could be obtained in the suckling animal, but the procurement of strictly quantitative data was not possible. It appeared that this resistance was related, at least in part, to the passive transfer of maternal antibody. A group of females that had recovered from an initial infection with the A59 virus was challenged 2 months later with 10^4 LD50 of virus. No outward

signs of disease were noted. The mice were mated 3 weeks after challenge. Test litters of various ages derived from such matings were inoculated with 10^3 LD50 of virus. Control litters of similar age were chosen at random from the BALB/c colony and inoculated with the same virus preparation. Animals that died during the first 2 days after inoculation were rejected. After 2 weeks of observation, survivors of all but 2 litters were killed and their livers were examined for visible lesions under a dissecting microscope. In table 1, the percent incidence of hepatitis determined by postmortem examination and the percent mortality are given for the various groups. Animals in all the control litters died with hepatitis. More than half the test animals inoculated with the challenge dose of virus during the 1st day of life survived 2 weeks. An increasing resistance to infection was observed when mice in successively older litters were challenged with virus. In any given litter in the older groups, the reaction of litter mates ranged from extensive liver necrosis terminating in death, through mild infection manifested by a few tiny areas of focal liver necrosis, to complete absence of any sign of disease.

TABLE 1.—Resistance of litters of previously infected parents to infection with 10^3 LD50 of MHV-A59

Litters*	Age (days)	Percent dead or missing	Percent with hepatitis†	Average day of death
Control	<1	100. 0	100. 0	6. 1
Test 1	<1	45. 4	100. 0	6. 8
Test 2	<1	42. 8	—	7. 1
Control	3	100. 0	100. 0	6. 8
Test 3	3	50. 0	80. 0	11. 5
Control	5	100. 0	100. 0	4. 8
Test 4	7	22. 2	14. 3	8. 5
Control	20	100. 0	100. 0	5. 4
Test 5	20	12. 5	—	7. 0

*Control litters chosen at random from the mouse colony; test litters born to and mothered by mice that had recovered from hepatitis.

†Values based on examination of dead animals and survivors killed 2 weeks post inoculation. Survivors of test litters 2 and 5 were not killed.

BALB/c mice less than 4 weeks of age usually died between the 3d and 7th days after virus inoculation. In older animals, deaths were less frequent. In one group of 66 weanling mice 4 weeks old, 65 percent died after injection of 10^3 LD50 of virus (titered in suckling mice). The same dilution of virus killed 18 percent of 78 animals 6 weeks of age, but none of 272 animals 8 to 13 weeks old. Death appeared to be more frequent in older animals when virus was injected intracerebrally, but insufficient data are available to permit significant comparison between the results obtained with the 2 routes of inoculation. The degree to which immunity resulting from inapparent infection, disseminated in the stock mice, influenced the resistance to virus in the older animals was not determined.

Blood smears were made from 15 splenectomized strain BALB/c mice and stained with Giemsa. No evidence of *E. coccoides* was observed in

tail bloods sampled on the 3d, 5th, 7th, or 10th days post splenectomy. In addition, smears made from 15 animals bearing serially transplanted leukemia were negative for *E. coccoides* on the 12th day after intraperitoneal implantation of the neoplasm. Two smears in the latter group were made from animals that had liver lesions characteristic of hepatitis infection. Other strains tested for susceptibility to the A59 virus were not checked for the presence of the blood parasite.

To determine the susceptibility of other strains to A59 virus infection, newborn and weaned C57BL, Swiss, A/He, A/HeN, A/LN, and C3H mice were inoculated with $10^{4.4}$ LD50 of BALB/c mouse-passaged virus. One week later, survivors were examined for liver lesions. The results presented in table 2 indicate that the virus could infect animals of each strain tested. No significance can be attached to the variation in response noted among the strains, because the presence or absence of factors, such as *E. coccoides* or humoral antibody that could influence the course of the disease, were not previously ascertained. In addition, it would be unreasonable to consider the A59 virus to produce latent infection solely in strain BALB/c mice.

TABLE 2.—Susceptibility of mice of various strains to infection with MHV-A59

Strain	Age	Number of litters	D/T*	I/E†	Average day of death
C57BL	Newborn	2	10/10	1/1	4.7
C57BL	4 Weeks	Mixed	5/6	6/6	5.6
Swiss	Newborn	3	17/18	12/12	4.9
Swiss	4 Weeks	Mixed	6/6	6/6	6.2
Swiss	5 Weeks	Mixed	6/24	13/24	5.0
A/LN	Newborn	3	20/24	9/9	6.5
A/LN	4 Weeks	Mixed	0/6	6/6	—
A/HeN	4 Weeks	Mixed	1/8	8/8	7.0
A/He	6 Weeks	Mixed	11/16	16/16	5.7
C3H	Newborn	3	3/8	3/6	6.3
C3H	4 Weeks	Mixed	1/6	3/6	7.0

*Numerator: number of mice that died during 7 days after injection of $10^{4.4}$ LD50 of BALB/c mouse-passaged virus; denominator: total number of mice inoculated.

†Numerator: number of mice with liver lesions; denominator: total number of mice examined.

Tenfold concentrates of A59 virus prepared by high-speed centrifugation were injected into newborn Fischer rats by the intraperitoneal or intracerebral route. No evidence of disease was observed. Similar inoculation of suckling hamsters failed to result in disease. There was no visual evidence of virus activity during 6 serial passages of MHV-A59 in the yolk sac or during 4 blind passages on the chorio-allantoic membrane of the embryonated hen's egg. Tubes of ML cells that were inoculated with extracts of yolk sac or extracts of chorio-allantoic membranes from each passage were negative, which indicated that there had been no virus propagation in either tissue.

Characteristics of A59 Virus Infection *In Vitro*

Morphological characteristics.—ML cell cultures were infected with an extract of diseased livers from mice in which the fifth animal passage of

A59 virus had been made. A portion of the fluids from these cultures was passed to fresh ML cultures 4 days later. In similar fashion, 25 passages were made at intervals of 3 to 6 days. Cytopathic changes observed in infected cultures consisted of small, dark cell elements loosely adherent to the surface of the glass and the surface of globose masses of polynucleated cell material. Tests indicated that the ML cultures used for these passages were contaminated with pleuropneumonia-like organisms (PPLO). Virus-infected fluids were treated at 4° C with anesthetic ether to eliminate the PPLO. When ML cells, maintained in an antibiotic-free medium and known to be free of extraneous agents, were infected with the ether-treated virus, large, polynucleated, protoplasmic masses (syncytia) were formed in the cultures. Uninfected ML cultures are shown in figure 6. The syncytia observed in ML cells infected with MHV-A59 are illustrated in figures 7 and 8. Five additional passages were made with the ether-treated virus, which made a total of 30 passages in ML cells. No change in virulence for suckling mice was observed in the virus after ether treatment and tissue-culture passage.

Syncytia were also produced in primary mouse-spleen cultures infected with the agent. The predominantly ameboid cell population in spleen cultures is illustrated in figure 9. The appearance of such a culture 3 days after addition of virus is shown in figure 10. Spindle cells, spared by the virus, succeed the ameboid cells destroyed by the virus and repopulate the surface of the glass. No cytopathic changes were produced, nor did virus persist, in cultures of Fischer rat spleen, rabbit kidney, monkey kidney, or HeLa cells.

Biological response to the virus.—During the initial attempts to study the virus quantitatively *in vitro*, erratic results were obtained. This was due to contamination of the cultures with PPLO, for reproducible titers were obtained after the elimination of the extraneous organisms. In the PPLO-contaminated system, infected cultures of ML cells that initially showed cytopathic changes sometimes recovered. Such cultures continued to produce virus. Transfer of fluids from a carrier culture to a fresh ML culture produced the syncytia typical of MHV-A59 activity. A number of the carrier culture tubes was cultivated for more than a year. Virus was lost from several cultures, but in most tubes infective virus was present throughout the observation period. The role of the PPLO contaminant in the establishment of the carrier state was not determined.

Titrations *in vitro* were made on 3 virus preparations. Two consisted of infected culture medium representing the 27th and the 29th passages of A59 virus in ML cells. The third preparation contained 30th-passage virus that had been sedimented from culture fluid by high-speed centrifugation and then resuspended in a saline diluent. The diluent consisted of 2 percent horse serum and 0.001 percent hyaluronidase in saline buffered at *p*H 6.8 with 0.01 M citrate. Each of a number of serial twofold dilutions of virus was inoculated into 40 ml culture tubes. During incubation a few culture tubes were discarded because they were contaminated or of poor quality. The results of these titrations are shown in table 3.

The goodness of fit with the first term of the Poisson distribution was estimated by the method recommended by Haldane (*21*). The observed values show good agreement with the one-particle curve. Log LD50 values based on the estimates of the number of infective units per ml determined for virus passages 27, 29, and 30 were 4.65, 5.82, and 5.72, respectively, with a standard error of approximately ± 0.05 log unit in each. The minimum number of tubes for titration of A59 virus required for any limits of error may be estimated approximately by the simplified method described by Bryan (*22*) [*see* equations (1b) and (3d)].

Serum neutralization of virus.—Serums from 24 litters of apparently healthy BALB/c mice ranging in age from 3 weeks to 3 months were tested *in vitro* for neutralizing antibodies against the A59 virus. Neutralization titers of 4 were obtained with 33 percent, and titers of 16 with 21 percent of the serums tested. No significant neutralization was produced by 46 percent of the serums. The antibody titers were not related to the age of the mice.

Similar tests were made with serums from uninoculated strain BALB/c mothers that had nursed experimentally infected litters. Titers ranging from 16 to 1,024 were observed in 5 serum specimens obtained from 2 weeks to 2 months after such secondary exposure to virus. When adult animals that recovered from an injection of virus were given a second injection and bled 7 days later, neutralizing titers of 256 were generally obtained.

Guinea pigs were inoculated subcutaneously with 1.0 ml of a mixture of equal parts of virus-infected ML culture fluids and Freund's adjuvant. During the succeeding 3 weeks, intraperitoneal injections of virus alone were made twice weekly. One month after the last injection, a final dose of virus was inoculated and the animals were bled 3 days later. A serum pool from 6 animals had a neutralizing titer of 16 against A59 virus. A control serum pool from a similar number of guinea pigs that had received uninfected ML culture fluids had no virus-neutralizing activity. Neutralization titers no higher than 4 were obtained in 3 rabbits subjected to a similar series of injections.

General Properties of A59 Virus

The A59 virus passed through Selas filters of 03 porosity and Millipore type-HA filter discs of 0.45 μ pore size. The agent could be sedimented in a Spinco Model L centrifuge. After 30 minutes at 40,000 rpm the supernatant fluid showed a drop in infectivity for ML cells by more than 3 log intervals. A suspension of the pellet in saline showed no significant loss of infectivity as compared with the initial extract. No sensitivity to 100 units of penicillin or 100 μg of streptomycin per ml of virus suspension was noted, nor did 50 μg per ml of aureomycin interfere with virus activity. When virus suspensions were saturated with anesthetic ether and stored at 4° C overnight, the titers determined in animals and in tissue cultures were about 1 log less than untreated virus controls similarly

TABLE 3.—Titration of MHV-A59; comparison of observed with expected frequencies

Virus passage 27					Virus passage 29					Virus passage 30				
Log dilution*	a	b	b′	d′	Log dilution*	a	b	b′	d	Log dilution*	a	b	b′	d
−3.9	40	0	0.80	2.45	−5.0	39	0	0.37	−1.72	−5.0	36	4	0.96	0.17
−4.2	36	4	5.65		−5.3	34	6	3.91		−5.3	37	3	6.21	
−4.5	25	15	15.03	0.03	−5.6	29	11	12.51	1.51	−5.6	24	16	15.77	−0.23
−4.8	13	27	24.53	−2.47	−5.9	18	22	22.37	0.37	−5.9	18	22	25.11	3.11
−5.1	5	34	30.52	−3.48	−6.2	10	30	29.91	−0.09	−6.2	9	31	31.70	0.70
−5.4	6	34	35.40	1.40	−6.5	5	35	34.59	−0.41	−6.5	5	35	35.59	0.59
−5.7	1	39	37.63	−1.37	−6.8	1	39	37.20	−1.80	−6.8	3	37	37.74	0.74
−6.0	1	34	33.95	−0.05	−7.1	3	37	38.57	2.87	−7.1	0	40	38.87	−1.13
					−7.4	2	37	38.30						
			$\chi^2 = 4.81$					$\chi^2 = 6.27$					$\chi^2 = 2.62$	
			$P = 0.57$					$P = 0.40$					$P = 0.76$	

*Final dilution in 1 ml of medium.

†a = Observed number of positive cultures; b = observed number of negative cultures; b′ = expected number of negative cultures calculated from the first term of the Poisson distribution; d = b′−b. Total number of tubes for each dilution = a + b.

stored. Heating the suspensions at 56° C for 30 minutes destroyed infectivity for animals and ML cells. Storage overnight at 4° C or in a dry-ice chest for 7 months did not alter the titer of virus suspended in tissue-culture fluids. A similar virus suspension was frozen and thawed 6 times by alternate immersion in a dry ice-alcohol bath and a water bath at 37° C without significant loss in titer.

Relationship to Other Viruses

Frozen-dried preparations of MHV-1 and MHV(Pr 1) mouse hepatitis viruses, representing the "hepatoencephalitis" group (*9*) were obtained through the courtesy of Dr. A. W. Gledhill [3] and Dr. J. B. Nelson.[4] The H747 virus was supplied by Dr. J. A. Morris.[5] Pooled virus of each type prepared in suckling Swiss mice was inoculated into tube cultures of mouse spleen and ML cells. Virus activity was evident only in cultures inoculated with MHV(Pr 1). Fluids from the cultures infected with this virus remained infective for weeks, which indicated that a carrier state was readily established. When the Pr 1 agent was serially passaged in ML cells, the infection caused cells to slough from the glass in loose clusters of rounded, dark elements. Occasional small syncytia were evident. The transfer of fluids from carrier cultures into fresh ML cultures resulted in the production of syncytia similar to those produced by the A59 virus. Overnight exposure of the Pr 1 agent to anesthetic ether at 4° C resulted in a 3 log drop in infective titer, as determined by assays *in vitro*. Guinea-pig antiserum to the A59 agent had a neutralization titer of 16 against both A59 and Pr 1 viruses. A BALB/c mouse serum that had a neutralizing titer of 64 against the homologous A59 virus had a titer of 16 against Pr 1 virus. It is evident, therefore, that there is a fairly close antigenic relationship between these two viruses.

For investigation of the relationship between the MHV-A59 and MHV-1 viruses, weanling Swiss mice were infected with liver extract containing the MHV-1 virus. After recovery, these mice were again inoculated with the same virus and bled 3 days later. Control animals were treated similarly with an extract of livers from uninfected suckling mice. The serums from 5 groups of about 5 mice each that had received the noninfectious extract showed no neutralizing activity *in vitro* against the A59 virus. Among the serums from 5 groups of mice that received Gledhill's MHV-1, one pool had no activity *in vitro* against the A59 virus. Two of the remaining pools had a titer of 4, another a titer of 16, and the last a titer of 64. Since cytopathic changes were not observed with MHV-1 virus *in vitro*, the last serum, (MHV-1)S 389, and one of the control serums, CMS 396, were used for cross-neutralization studies *in vivo*.

A pool of antiserum against the A59 virus was obtained from a group of strain BALB/c mice. Two months after an initial injection of virus these animals received a second injection of the agent. Three days later

[3] National Institute for Medical Research, Mill Hill, London.
[4] The Rockefeller Institute, New York 21, N.Y.
[5] National Institutes of Health, Bethesda, Md.

they were bled. The pooled serum, (A59)S 365, had a neutralization titer of 64 against the homologous virus *in vitro*. A normal BALB/c mouse serum without neutralizing activity, NMS 366, was used as control. Phosphate-buffered saline at *p*H 7.25 served as diluent (*23*). Mixtures of diluted serum and appropriate dilutions of either A59 or MHV-1 virus were injected into suckling mice by the intracerebral route, because previous experience had shown that the intraperitoneal route was unsatisfactory for the demonstration of virus neutralization. Two litters of strain BALB/c mice were used for each serum-virus mixture. Since the results of 2 such tests were similar, the data were pooled and are presented in table 4. Also shown are the results obtained without serum, with serum CMS 396, and with serum (MHV-1)S 389. The antiserum against A59 virus provided protection *in vivo* against the homologous virus and gave slight activity against the MHV-1 virus. Serum from mice exposed to MHV-1 infection was not effective against the homologous virus. The slight activity obtained against the A59 virus was to be expected from the results obtained with this serum sample in the tests *in vitro*.

DISCUSSION

Although hepatitis occurred frequently in strain BALB/c mice bearing whole-cell transplants of the Moloney murine leukemia, the disease was seldom noted during studies with cell-free preparations of the leukemia agent. That more than one virus was responsible for the hepatic disease in our strain BALB/c stock animals was evidenced by fundamental differences in the behavior of 2 virus strains obtained from animals with liver lesions. The first, which was most active in suckling mice, did not grow in Earle's cloned ML cell line, NCTC 1469. In this respect, as well as in its lack of stability to ether treatment, it resembled Gledhill's MHV-1, isolated from Park's strain mice with spontaneous liver disease. The second isolate, MHV-A59, could be propagated and assayed quantitatively in ML cells. Although hepatitis viruses have been propagated in other tissue-culture systems (*24–26*), quantitative assays by methods *in vitro* have not been achieved heretofore.

A close relationship between the MHV-1, JHM, EHF120, and H747 viruses was established by Morris (*9*) using neutralization and complement-fixation techniques. Recently, Starr and Pollard (*27*) demonstrated cross-reactivity between the complement-fixing antigens of MHV-1 and MHV(Pr 1). Sufficient information to permit classification of the hepatitis agent of Braunsteiner and Friend (*1*) with the former group is lacking. Its reported stability to a temperature of 80° C for 30 minutes (*28*) suggests that it may be different. The hepatoencephalomyelitis virus isolated by Stanley and coworkers (*29*) has been related to the REO viruses (*30, 31*). Another agent that may be responsible for focal necrosis of the liver and spleen is ectromelia virus (*32*). Serums from mice that had recovered from A59 virus infections were examined by the hemag-

TABLE 4.—Neutralizing capacity of mouse serums against A59 and MHV-1 viruses determined in 1- to 4-day-old BALB/c mice inoculated intracerebrally

Virus	Serum*	Serum dilution	Virus dilutions					
			10^0		10^{-1}		10^{-2}	
			Deaths† (percent)	Liver lesions‡ (percent)	Deaths† (percent)	Liver lesions‡ (percent)	Deaths† (percent)	Liver lesions‡ (percent)
A59	None	Diluent	100. 0	100. 0	100. 0	100. 0	58. 1	80. 0
A59	NMS 366	1:16	96. 7	100. 0	99. 1	100. 0	N. T.§	N. T.
A59	(A59)S 365	1:16	21. 2	51. 7	12. 5	3. 5	N. T.	N. T.
MHV-1	None	Diluent	100. 0	96. 0	93. 6	100. 0	41. 4	57. 7
MHV-1	NMS 366	1:16	100. 0	100. 0	79. 4	100. 0	N. T.	N. T.
MHV-1	(A59)S 365	1:16	93. 1	95. 5	25. 9	70. 8	N. T.	N. T.
A59	None	Diluent	100. 0	100. 0	100. 0	100. 0	35. 3	14. 3
A59	CMS 396	1:8	100. 0	100. 0	68. 8	100. 0	N. T.	N. T.
A59	(MHV-1)S 389	1:8	94. 4	100. 0	53. 3	58. 3	N. T.	N. T.
MHV-1	None	Diluent	100. 0	87. 5	100. 0	100. 0	23. 5	56. 3
MHV-1	CMS 396	1:8	100. 0	100. 0	57. 1	100. 0	N. T.	N. T.
MHV-1	(MHV-1)S 389	1:8	100. 0	87. 5	58. 3	100. 0	N. T.	N. T.

*NMS = serum from untreated stock strain BALB/c mice; CMS = serum from control Swiss strain mice receiving injections of uninfected mouse-liver extract; (A59)S = serum from BALB/c mice that recovered from infection with MHV-A59; (MHV-1)S = serum from Swiss strain mice that recovered from infection with Gledhill's MHV-1.

†Values based on number of animals surviving 2 days post injection.

‡Values based on total number of animals examined after death or killed 7 days post infection.

§N. T. = not tested.

glutination-inhibition test at the Mouse Pox Laboratory, Seton Hall College of Medicine and Dentistry, Jersey City, New Jersey, and found to be negative with respect to ectromelia.

Neutralization tests conducted *in vitro* with serums from apparently normal litters of strain BALB/c mice demonstrated that neutralizing antibody against the A59 virus was present in a high percentage of the litters. In similar tests, a few serum pools obtained from small groups of uninfected weanling Swiss mice showed no neutralizing activity against the virus. Nevertheless, the susceptibility of Swiss mice to infection with the A59 virus indicates that pools of other strains of hepatitis virus prepared in these mice could be contaminated with the indigenous agent. Serums from Swiss mice that recovered from infection with MHV-1 did possess neutralizing activity against the A59 virus. However, no evidence of A59 virus activity was noted when ML cells were infected with the MHV-1 pool, and contamination of the latter with A59 virus was, therefore, considered unlikely.

The Pr 1 virus, which Nelson isolated from Pr mice bearing a transplanted lymphoid neoplasm, was carried through 2 suckling Swiss mouse passages followed by 7 passages in ML cultures. Neutralization of the tissue-culture-passaged virus by antiserums against A59 virus prepared both in mice and in guinea pigs was shown by tests *in vitro*. The necessity for animal passage was unfortunate, since the succeeding tissue-culture passages could be selective for a contaminant. However, Bang and Warwick (*33*) found that a virulent hepatitis virus obtained from Nelson, presumably MHV(Pr 1), destroyed macrophages migrating from explant cultures of Pr strain mouse livers. The ability to destroy cultured mouse macrophages is shared by MHV-A59. Differences in behavior between the viruses do exist, for Bang and Warwick demonstrated a high degree of resistance of strains C3H and A mice to infection with Nelson's virus. High resistance was not observed when these strains were infected with the A59 virus. It is concluded that MHV-A59 is a member of the "hepatoencephalitis" group of murine viruses and most closely related to the MHV(Pr 1) of Nelson.

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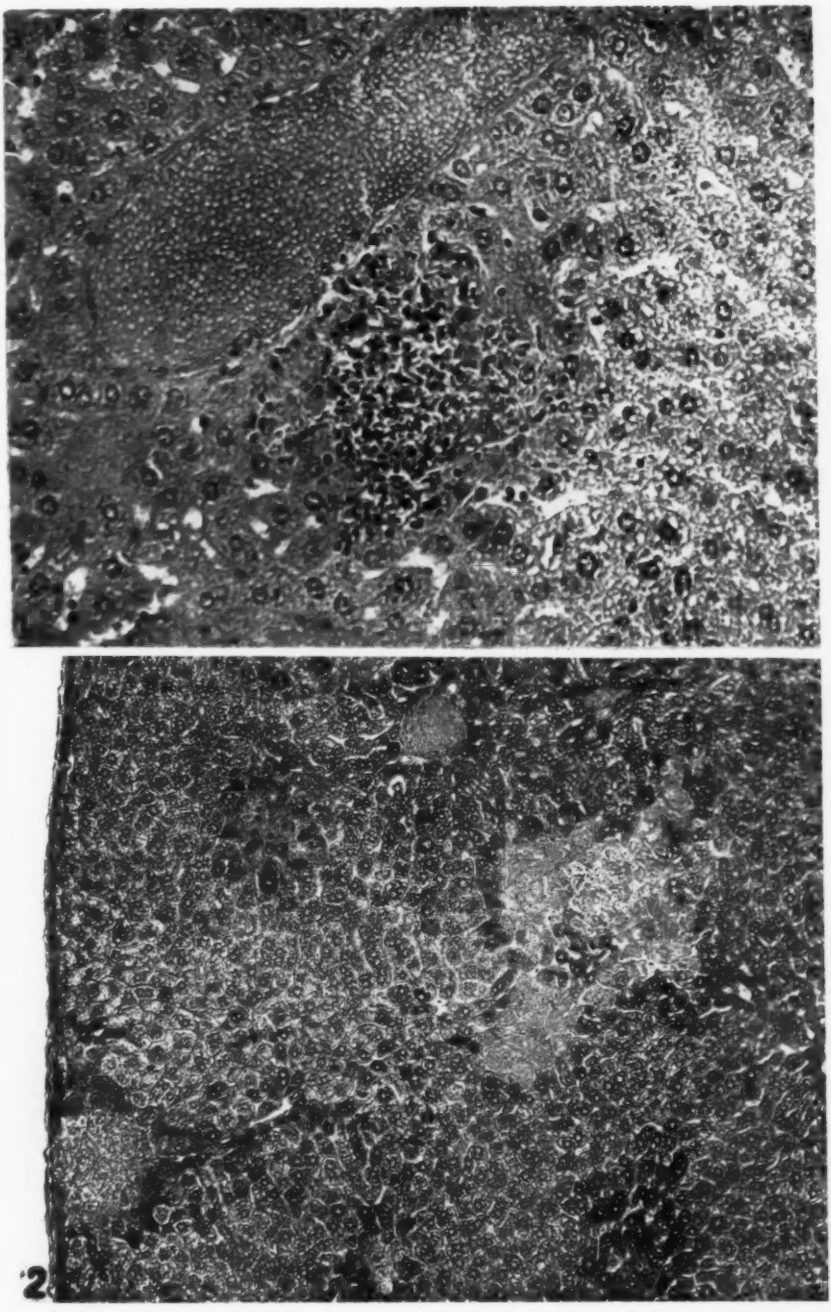


593953—61——7

PLATE 5

FIGURE 3.—Mouse liver 96 hours after injection of MHV-A59. *Note* diffuse distribution of hepatic necrosis with survival of cells adjacent to central vein. Giemsa. × 135

FIGURE 4.—Giant cells in mouse spleen 24 hours after injection of MHV-A59. Hematoxylin and eosin. × 210

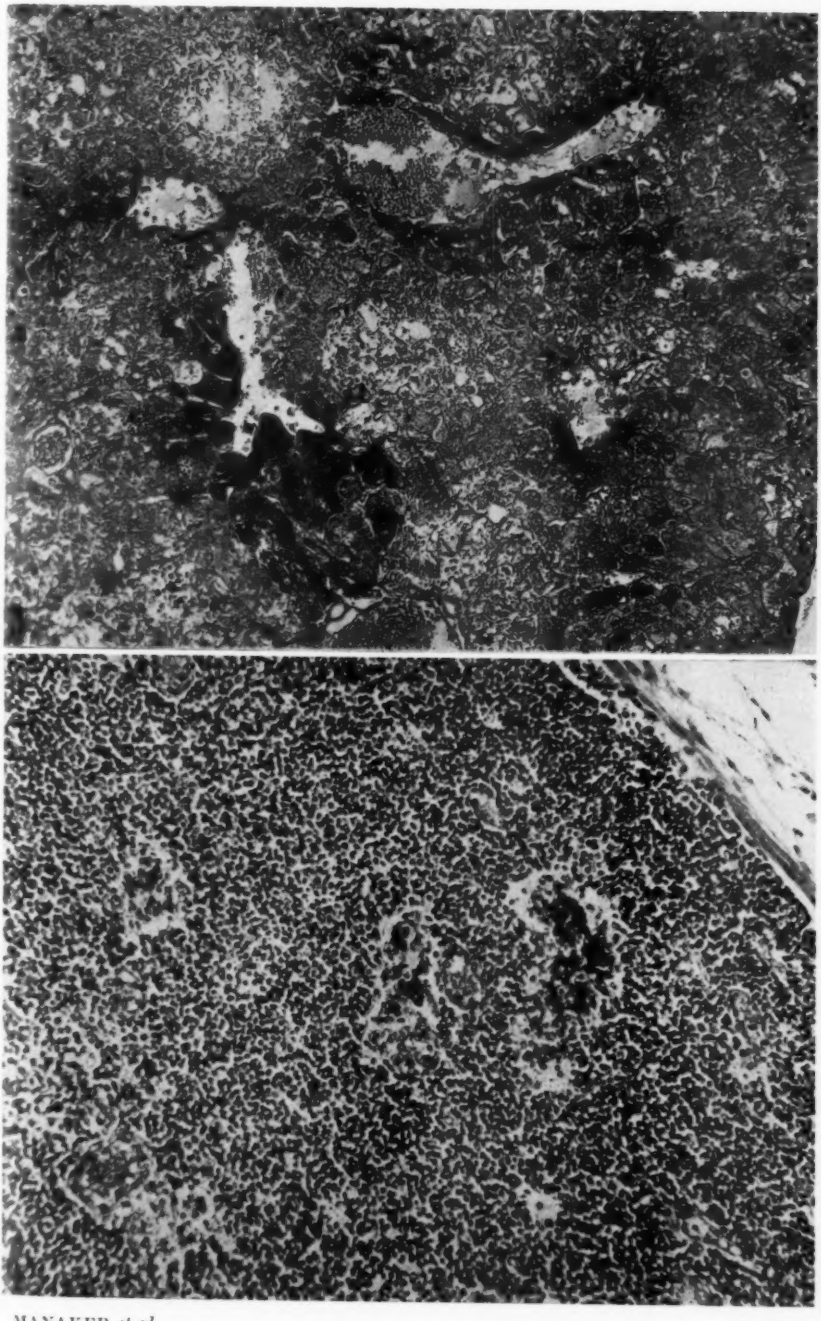


PLATE 6

FIGURE 5.—Perifollicular giant cells in mouse mesenteric lymph node 24 hours after injection of MHV-A59. Hematoxylin and eosin. × 210

FIGURE 6.—Uninfected culture of Earle's mouse-liver cell line, NCTC 1469. Unstained living cells. × 200

FIGURE 7.—Syncytia in a culture of Earle's mouse-liver cell line 24 hours after inoculation with 10^4 TCID50 of MHV-A59. Unstained living culture. × 75

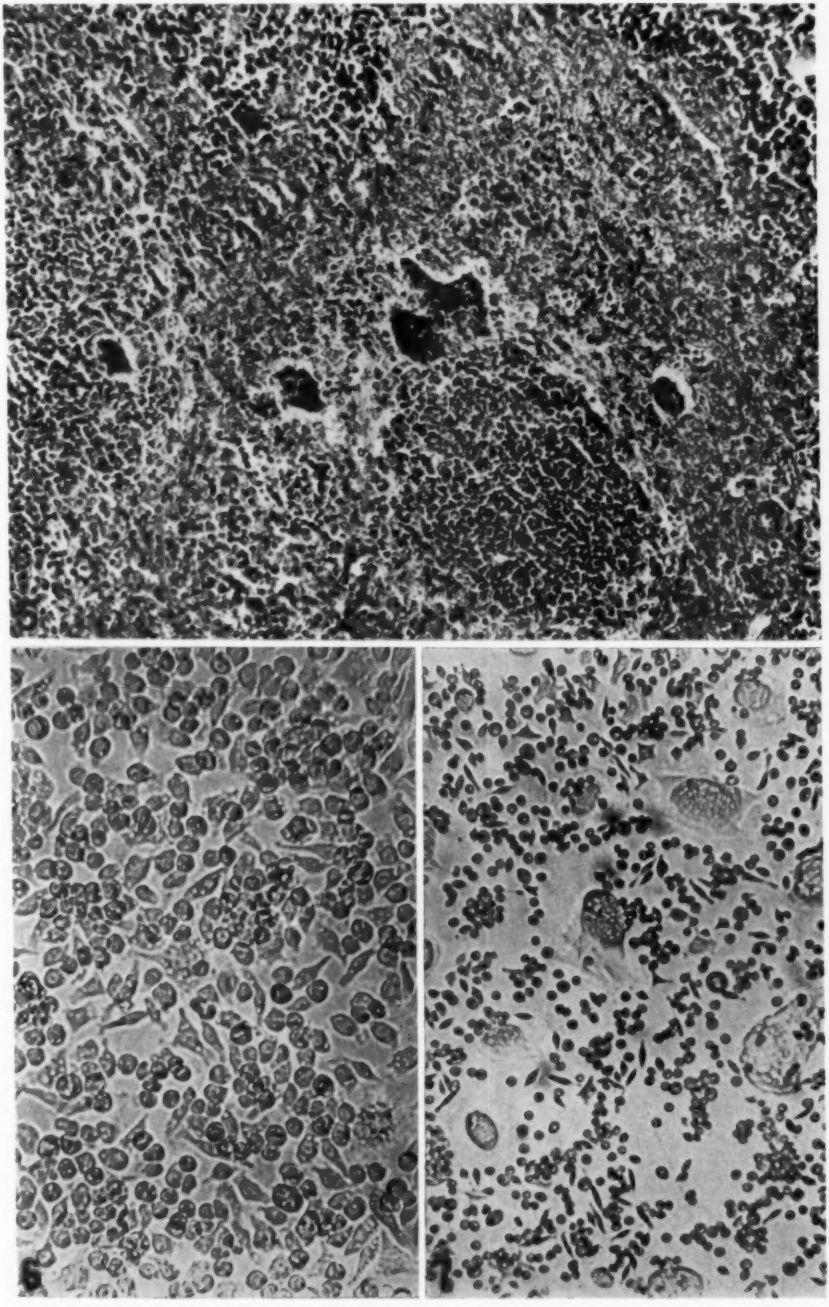
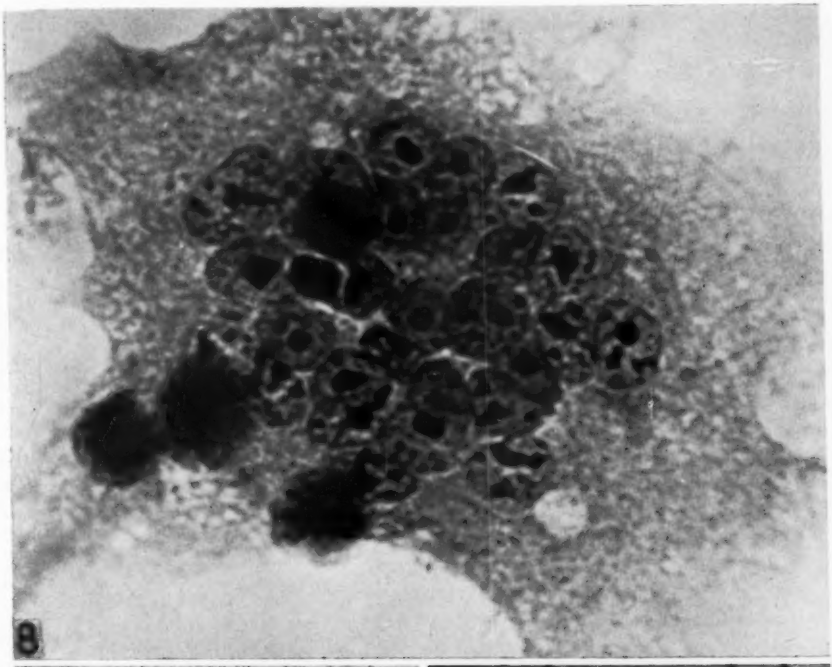


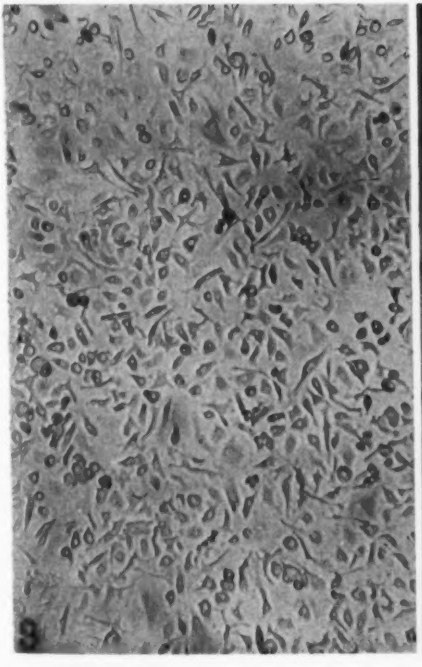
PLATE 7

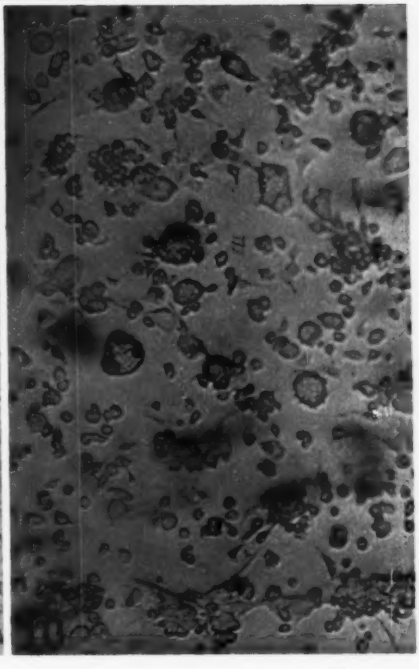
FIGURE 8.—Syncytium in a culture of Earle's mouse liver cell line infected with MHV-A59. Hematoxylin-Schorr S3. × 1,250

FIGURE 9.—Uninfected culture of mouse spleen. Unstained living cells. × 75

FIGURE 10.—Mouse spleen culture 3 days after inoculation with 10^2 $TCID_{50}$ of MHV-A59. Unstained living culture. × 75







Treatment of Total-Body X-Irradiated Monkeys With Autologous and Homologous Bone Marrow[1,2]

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SUMMARY

Rhesus monkeys were given injections of autologous or homologous bone marrow after total-body X irradiation. After doses of 650 r or more, untreated monkeys survived for 12 to 16 days without recovery of the peripheral blood counts. Five of 6 animals treated with 2.2 to 12.9 $\times$ 10^8 autologous bone marrow cells after radiation doses of 850 to 950 r showed hematopoietic recovery and survived for over 100 days. After lethal radiation doses and treatment with more than 8 $\times$ 10^8 homologous bone marrow cells, recovery of hematopoiesis was observed in 14 monkeys. In most of these male animals the proliferation of female donor cells was demonstrated by the presence of typical "drumsticks" in the polymorphonuclear granulocytes of the peripheral blood. However, only 2 animals survived more than 30 days, and the average survival time, 21.6 days, was only slightly longer than that of the controls. A syndrome of anorexia, diarrhea, and wasting, often combined with dermatitis or jaundice, occurred only in the animals treated with foreign bone marrow. From the similarity of this syndrome and the autopsy findings (described in the following paper) to those of secondary disease in rodents, it is concluded that the monkeys which, after irradiation, were treated with homologous bone marrow died from severe and early development of secondary disease. The implications of these findings are discussed.—J. Nat. Cancer Inst. 27: 53–65, 1961.

SEVERAL AUTHORS have reported varying degrees of success in attempts to prolong the life of irradiated monkeys or apes by postirradiation injection of bone marrow cells (*1–5*). These studies were concerned essentially with survival times and hematological data. In the broad range of work with irradiation and bone marrow transplantation in

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[2] The major part of this work was performed under contract with Euratom (European Atomic Energy Community) 51–53 rue Belliard, Brussels, Belgium.

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593953—61——8

various species during recent years, a number of new problems and phenomena has arisen which must be dealt with before these techniques can be applied efficiently to clinical trials. The present study was initiated 1) to see if similar phenomena result with the use of bone marrow transplantation in the primate, and 2) to compare such phenomena with those already known to occur in lower animals.

MATERIALS AND METHODS

Animals.—*Macaca mulatta* monkeys, maintained in a large stock colony, were caged separately or in small groups not exceeding 5 animals. They were fed a diet of fresh fruits and vegetables including cooked rice supplemented with various vitamins. The stock monkeys thrived and gained weight on this regimen according to a normal curve. The animals used in irradiation experiments had all been in the stock colony from 3 months to 1 year before they were used. A few weeks before irradiation the animals were caged separately and control hematological and bacteriological studies were done. Monkeys weighing between 2.8 and 4.8 kg were used for irradiation, and very young animals weighing between 2.0 and 3.0 kg were selected as bone marrow donors. The recipients of homologous bone marrow were all male and the donors female. All of the autologous recipients were female except 1. Every attempt was made to select healthy animals for irradiation, but this was extremely difficult to evaluate, as we will explain in the discussion of this paper.

Irradiation.—The irradiation facility consisted of a Philips X-ray machine operating at 200 kvp, 20 ma, and using 1.5 mm Cu filter (HVL 1.95 mm Cu). The monkeys were placed in a cylindrical Lucite cage 20 cm in diameter with a height adjustable between 28 and 36 cm. The cage was turned in the center of the X-ray beam 6 times/minute by an electric motor. The target distance to the center of the cage was always 70 cm. Under these conditions the tissue dose rate was approximately 18 r/minute.

Ionization chamber measurements showed that the dose was homogenously distributed in a cylindrical Masonite phantom. The radiation doses were obtained by correction of the phantom dose for variations caused by the different body weights of monkeys. These corrections were calculated for various average monkey diameters on the basis of the depth-dose curves obtained in the Masonite phantom.

Bone marrow treatment.—The donor animals were anesthetized with 25 mg/kg body-weight Pentothal sodium and anesthesia was continued with drip ether. A trocar 12 cm long with a 2 mm inside diameter was forced into the knee joint and hammered into the epiphysis of the femur toward the proximal end of the bone. When the point entered the marrow cavity, a syringe washed with heparinized Tyrode's solution was fitted to the trocar, and bone marrow mixed with whole blood was pulled into the syringe. The trocar was pushed a few mm further and again a sample

was taken. This process was continued for the entire length of the marrow cavity. The syringes were emptied into a few ml of Tyrode's solution and the suspension was filtered through layers of nylon gauze. From this suspension samples were taken to determine total and eosin-resistant cell counts. (An average of 93% was eosin-resistant.) Numbers of cells mentioned in this paper refer always to total number of eosin-resistant bone marrow cells.

Treatment and handling.—All monkeys were fasted for 24 hours preceding irradiation or operation.

In the autologous experiments the bone marrow was first removed, the animal was irradiated after recovering from anesthesia, and the bone marrow was injected within 1 to 2 hours. In the homologous experiments the bone marrow was injected 24 hours after irradiation unless otherwise stated. In each animal the bone marrow suspension was injected into the short saphenous vein at the rate of about 2 ml/minute, with a syringe or with a continuous drip infusion set, depending upon the volume of suspension.

Immediately after irradiation the animals, except #6 and 8, were housed in a room separate from the stock colony; this room was strictly quarantined and personnel were required to wear a gown, cap, mask, and rubber shoes before entering the room. The same diet was fed irradiated monkeys and stock animals except during periods of anorexia when, in most cases, a vitamin-supplemented milk diet was supplied by stomach tube.

Four different antibiotics were used alternately and were given intramuscularly in the following doses: penicillin, 400,000 units/day often combined with 250 mg/day of streptomycin; or terramycin,[8] 100 mg/day; or chloramphenicol, 250 mg/day. Other symptomatic treatment included the use of tincture of opium for diarrhea, in addition to parenteral fluid therapy (Ringer's solution, normal saline, or 5 percent glucose in normal saline). Ulcers in the mouth were cleansed with potassium permanganate solution and the eyes were treated with boric-acid solution when they became inflamed. Some monkeys were killed by intravenous administration of Pentothal sodium a few hours before the estimated time of spontaneous death. The reasons for this procedure were twofold: 1) to avoid unnecessary suffering of the animals; 2) to permit procurement of fresh autopsy materials.

Hematology.—Blood samples were taken 2 or 3 times before irradiation and 2 or 3 times a week thereafter until the peripheral blood counts returned to normal or the animal died. Determinations were made for total erythrocytes, reticulocytes, thrombocytes and leukocytes, leukocyte differential, and hematocrit.

In the homologous-treated animals the neutrophil "drumstick" count was also determined from each blood sample to ascertain the presence of donor-type cells in the peripheral blood of the host. This procedure was similar to that of Porter studying irradiated, bone-marrow-treated

[8] A generous gift of terramycin was obtained from Pfizer N. V., the Netherlands, through the courtesy of Mr. N. Olivier and Mr. J. A. Eliasar.

rabbits (*6*). Two types of smears were made to determine the drumstick count, 1 of whole blood and 1 of concentrated leukocytes. The slides were fixed in methanol and stained in a 0.5 percent solution of pinacyanole in 70 percent methanol. Whenever possible, 1,000 mature neutrophil leukocytes were counted to determine the percentage showing the characteristic appendage of the female cell. In the male monkeys, of 140 preirradiation slides none showed 1 percent or more drumsticks (90 percent showed less than 0.3 percent). In the female donor monkeys none of the slides showed less than 2 percent.

The limitations of this method for the recognition of proliferating donor cells are obvious: Mixtures of host and donor cells, if present, cannot be recognized, and our definition of a take must imply that at some time after irradiation at least a fraction of the recovery of hematopoiesis was due to proliferation of donor cells. Similarly, absence of a take cannot be proved, since a low drumstick count on the regenerated polynuclears could theoretically be caused by a small fraction of donor cells plus a large fraction of host cells. We had additional difficulties in applying this method because some animals, with regeneration of their peripheral blood, survived for only short periods. In these cases it was sometimes impossible to check 1,000 mature polynuclears, since the peripheral blood in early regeneration showed predominantly immature cells of the myeloid series.

In the light of these difficulties we established the following categories to assess a take of donor cells: [9]

Take, i.e., *proliferation of donor cells:* if, in counts of at least 1,000 mature polynuclears at any time more than 6 days after irradiation, significantly ($P<0.01$) higher drumstick percentages were found than expected on the basis of the combined preirradiation counts.

Probable take: if the combined checks in the period of postirradiation hematopoietic recovery showed a higher percentage of drumstick-bearing cells than were found in the preirradiation period (but without statistical significance).

Insufficient data: if, as was the only conclusion in two animals during postirradiation hematopoietic recovery, smears showed less than 1,000 mature polynuclears.

No take: 1) if no hematopoietic recovery occurred on the basis of peripheral blood counts and autopsy data, or 2) if hematopoietic recovery occurred, but when only during a short period of this recovery the drumstick counts were consistent with the possibility of a take. (This occurred in a single animal.) Later the drumstick counts were so consistently low as to be compatible only with the possibility of either complete repopulation by the host cells, or of only a very small fraction of donor cells surviving.

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[9] This formal classification was established on the basis of the statistical evidence from the drumstick data only. On the basis of additional suggestive data, notably the time of recovery of hematopoiesis combined with the radiation doses given, the authors are convinced that, in the animals mentioned in the category "Insufficient Data," a take of the donor cells occurred; however, since proof of this conviction is lacking, the formal classification is used in the discussion of the results.

RESULTS

The radiation doses, numbers of bone marrow cells, and results are given in tables 1, 2, and 3.

Table 1.—Irradiated control monkeys *

Monkey No.	Sex	X-ray dose (r)	Antibiotics†	Anorexia (began on day)	Diarrhea (began on day)	Reticulocytes (>1% between days)	Leukocytes (>1000/-mm³ between days)	Survival time (days)
31	♂	550	PSC	—	—	—	—	15
35	♂	550	PSCT	—	—	20–22	18–20	>100
39	♂	650	PS	—	—	—	—	14
63	♂	650	T	—	—	—	—	15
5	♀	700	—	—	—	—	—	16
52	♂	810	PTS	—	—	—	—	15
17	♂	910	PS	11	6	—	—	15
3	♀	1050	—	—	6	—	—	12

*None suffered from eye inflammation, jaundice, dermatitis, or mouth lesions. All showed either petechiae or major hemorrhage.

†P = penicillin; S = streptomycin; T = terramycin; C = chloramphenicol.

Survival Times

All the control monkeys (table 1) irradiated with 650 r or more died between 12 and 16 days after irradiation without evidence of hematopoietic recovery. One of the 2 monkeys irradiated with 550 r survived over 100 days with complete recovery of hematopoiesis.

Six of the 7 animals, which received a suspension of their own bone marrow after irradiation, survived over 30 days (table 2). In fact, 2 animals have survived for more than a year and a total of 4 in the group are still alive at the time of writing. One animal, which received the highest radiation dose given any monkey in the experiment, survived for only 6 days; this was a shorter lifespan than that of any animal, including the controls.

Three of the 18 homologous-treated monkeys survived 30 days after irradiation (table 3). Animal #30 is still alive at the time of writing, over 200 days post irradiation, but the dose administered was less than an LD100. Monkey #57 survived 47 days and #43, 31 days after irradiation and treatment. Excluding animal #30, since it received sublethal irradiation, the mean survival time for the homologous group was 20.5 days. There was a decided difference in mean survival times of the animals showing a take of the bone marrow graft and those which did not. The 10 animals that showed a take (*see* page 56) had a mean survival time of 24.4 days, while the 3 that did not accept the graft had a mean survival time of 15.3 days. It is not impossible that the animals in the categories "Probable Take" (2 animals) and "Insufficient Data" (2 animals) had, in fact, takes of donor tissue but they did not survive long enough to permit this conclusion to be drawn. The average survival time of all

Table 2.—Irradiated monkeys treated with autologous bone marrow*

Monkey No.	Sex	X-ray dose (r)	Viable bone marrow cells injected ($\times 10^6$)	Antibiotics†	Anorexia (began on day)	Diarrhea (began on day)	Petechiae	Reticulocytes (>1% between days)	Leukocytes (>1000/mm³ between days)	Survival time (days)
48	♀	850	5.0	S	7	7	—	14–17	14–17	>200
45	♂	850	12.9	T	—	—	—	8–11	8–11	>270
16	♀	870	8.7	PSTC	7	7	+	17–20	17–20	>370
12	♀	905	2.7	PSTC	—	—	+	—	—	50
11	♀	905	5.6	PSTC	—	—	+	13–15	13–15	163
33	♀	925	2.2	—	—	—	+	20–22	13–15	>500
40	♀	1065	2.7	P	—	—	—	—	—	6

*None suffered from eye inflammation, jaundice, dermatitis, or mouth lesions.
†P = penicillin; S = streptomycin; T = terramycin; C = chloramphenicol.

Table 3.—Irradiated monkeys treated with homologous bone marrow

Monkey No.	Sex	X-ray dose(r)	Viable bone marrow cells injected ($\times 10^8$)	Antibiotics*	Anorexia (began on day)	Diarrhea (began on day)	Eye inflammation	Dermatitis	Jaundice	Mouth lesions	Petechiae	Donor leukocytes†	Reticulocytes (>1% between days)	Leukocytes (>1000/mm³ between days)	Survival time (days)
30	♂	550	25. 2	PST	7	7	—	—	—	—	+	NT	15–20	12–15	>200
46	♂	600	15. 2	PST	11	17	—	+	+	—	+	T	11–14	7–9	19
57	♂	625	22. 0	PSTC	—	—	+	+	+	—	—	T	12–14	12–14	47
28	♂	650	21. 5	STC	—	11	+	+	+	—	—	PT	11–14	9–11	14
43	♂	650	24. 8	TC	7	5	—	+	—	—	+	T	10–13	8–10	31
27	♂	650	26. 2	T	15	13	—	+	+	—	+	T	9–12	7–9	19
44	♂	650	37. 4	C	5	3	—	—	—	—	—	PT	—	6–8	10
15	♂	650	47. 8	T	7	6	—	+	—	—	—	T	—	8–11	11
51	♂	705	11. 2	PST	22	8	+	+	—	—	+	T	13–18	9–11	26
56	♂	740	24. 0	PSTC	8	17	+	+	—	+	+	T	15–18	8–11	23
54	♂	755	13. 9	PSC	9	—	—	?‡	—	—	+	T	12–15	6–12	19
37	♂	795	7. 9	P	—	—	—	?	—	+	+	NT	—	—	17
53	♂	805	20. 0	PSTC	7	4	+	—	+	+	+	T	11–14	9–11	22
8	♂	855	8. 0	PS	5	5	+	?	—	+	+	NT	—	—	13
6	♂	865	8. 5	PSTC	9	6	—	?	+	+	+	T	10–19	10–19	27
32	♂	905	9. 5	P	8	6	+	?	+	+	+	ID	7–14	14–19	21
38	♂	915	13. 7	PST	7	5	—	?	+	—	+	ID	—	7–14	14
41	♂	935	7. 1 5. 0§	P	9	5	—	?	—	+	+	NT	—	—	16

*P = Penicillin; S = streptomycin; T = terramycin; C = chloramphenicol.

† T = Take of graft; PT = probable take; NT = no take of graft; ID = insufficient data; (*see* text Materials and Methods).

‡? = Not noted.

§ The second bone marrow injection was given 5 days post irradiation.

animals except the "No Takes" (21.6 days) might be more appropriate as an expression of the results of successful homologous transplantation. The mean survival time of the 6 control animals irradiated with lethal doses was 14.5 days, which was comparable to that of the animals showing no take of the bone marrow graft. Finally, the 2 animals that survived for a shorter period than any of the control animals received the highest number of bone marrow cells.

Hematology

The speed of recovery of the peripheral blood in the autologous-treated animals appears to be related to the number of bone marrow cells injected after irradiation as the data in table 2 show. Of the 3 animals receiving less than 3.0×10^8 viable bone marrow cells, only 1 showed recovery of the peripheral blood elements, this being relatively late for the reticulocytes. Animal #45, which received the largest number of cells, showed the earliest recovery of both reticulocytes and leukocytes. Animal #40 showed no indication of recovery of hematopoietic function and apparently died from other causes before the usual time for repopulation of the peripheral blood to begin. The other similar case (animal #12) was unusual in that it survived 50 days, with the leukocyte count never returning above $1000/mm^3$ and the reticulocytes never reaching 1 percent. The thrombocytes also remained at very low levels throughout the 50 days. The other animals showed a rapid return of the peripheral blood values to normal, once hematopoiesis had begun.

In the animals treated with homologous bone marrow the leukocyte counts in most cases returned toward normal sooner than reticulocyte counts. The thrombocytes in the animals with other indications of recovery also showed a postirradiation increase, though this was usually much later than the return of leukocytes and reticulocytes. Animal #30, which was irradiated at a sublethal dose level and showed evidence of recovery of its own bone marrow, was among the slowest to recover its peripheral blood.

Three animals treated with homologous bone marrow showed a marked leukocytosis during their postirradiation recovery period. The highest white blood count was 85,600 in monkey #6 on the 19th day after irradiation. In the animals that received a lethal dose of irradiation there appears again to be a correlation between the number of bone marrow cells injected and the speed of regeneration of the peripheral blood: Among the animals which received 10^9 or less cells, recovery of hematopoiesis was either absent or occurred some time after irradiation.

Clinical Observations

Many animals showed severe weight loss after lethal doses of radiation. In the controls and after autologous-bone-marrow treatment, this amounted to 0.74 percent and 0.72 percent, respectively, of the initial body weight/day during the first 12 to 16 days after irradiation. In the homologous-bone-marrow-treated animals showing a take or a probable

take, the comparable loss was 0.93 percent. However, comparison of later weight loss in the surviving animals showed a marked average further loss of weight in the surviving homologous animals (1.23 percent/day), whereas in the autologous animals in a comparable period the average weight *increase* was 0.34 percent of the initial weight per day.

The total weight loss in the homologous animals was often as much as ¼ to ⅓ of the original weight. In 1 or 2 instances it was possible to prevent excessive loss of body weight by tube feeding or by administration of parenteral fluids; however, this was apparently a transient phenomenon. The loss of weight is attributed to anorexia and diarrhea in most cases, but these factors cannot account for the loss in all animals; *e.g.*, the weight of animal #57 went from 4,360 g original weight to 2,980 g at death, though the animal never had anorexia or diarrhea during the postirradiation period. Excessive loss of weight was obviously an important factor contributing to the death of many animals.

Eighteen of the 25 irradiated, bone-marrow-treated animals showed some visible indication of hemorrhage. This was usually in the form of petechiae in the skin, though some animals showed massive hemorrhage from the nose, rectum, or eye. When the thrombocytes were lowest in number in the peripheral blood, it was sometimes difficult to prevent bleeding after an intravenous or intramuscular injection.

There appears to be a decided difference in the occurrence of diarrhea in the animals treated with autologous and homologous bone marrow. Only 2 of the 7 autologous animals had diarrhea during the experiment, while 15 of the 18 homologous animals had it. Most of the diarrhea in the homologous animals was almost impossible to control. In very few can it be shown that the tincture of opium or the antibiotics helped to overcome the condition. Although the feces of an animal in this condition were always cultured, a pathogenic organism was seldom demonstrable.

Nearly always the occurrence of anorexia paralleled that of diarrhea. Only 1 animal had diarrhea without accompanying anorexia and vice versa. In most cases the anorexia followed the diarrhea by a few days, or began the same day. Attempts to make the food more palatable were unsuccessful in overcoming this condition. The duration of the anorexia and diarrhea usually corresponded.

None of the autologous-bone-marrow-treated animals showed eye inflammation, mouth lesions, or jaundice, though these were common in the homologous-treated animals (*see* table 3). Eye inflammation consisted of a reddening of the eyes and lids and the production of a heavy, brownish-green exudate which sometimes tightly shut the lids.

Data on ulceration and necrosis of the gingiva and ulcers in the corners of the mouth are given in table 3 under the heading "Mouth lesions." Seven animals became jaundiced, usually 3 to 4 days before death.

In some homologous animals a characteristic skin lesion developed. After this was noticed the first time, it was recognized clinically in 8 of 10 monkeys (*see* table 3). Characteristically, in the 2d week after irradiation an erythema developed over the face and the anterior part of the chest.

This erythema was confluent in a few days and became a uniform erythrodermia that gradually decreased in intensity. In the 3d week after irradiation, the skin became scaly in the same area and the redness had disappeared completely. This lesion was distinctly different from an irradiation erythema that sometimes appeared on the faces of the animals the 2d or 3d day after irradiation and subsided after a few days. The latter erythema was also seen in some of the control and autologous animals, while the former type appeared only in the animals treated with homologous bone marrow.

DISCUSSION

One of the most important problems in this study was the lack of availability of noninfected, healthy animals for irradiation and bone marrow treatment. Although the monkeys were quarantined from 3 months to 1 year before handling, it was evident at autopsy that some were not completely free from parasitic infestation at the time of irradiation. A large percentage carried an intestinal parasite, *Œsophagostomum apiostomum*. Although treatment apparently clears this parasite from the lumen of the intestine, an infestation can remain, since the worm burrows through the mucosa and settles into a nodule in the intestinal wall. This then becomes a hazardous source of infection from the intestine, especially when the animal is faced with a stress such as irradiation, and is only one example of any number of conditions or states of disease which might exist in patients, either those receiving irradiation accidentally or those in whom it is used therapeutically. This will undoubtedly create a problem in the clinical use of bone marrow transplantation.

The entire picture of the autologous-bone-marrow-treated animals is in strong contrast to that of the homologous-treated animals. Six of the 7 autologous monkeys survived over 30 days after irradiation and treatment. Animal #40, which showed no evidence of regenerating bone marrow, received the highest X-ray dose, 1065 r. Death occurred on the 6th day and the autopsy findings (*7*) showed that the animal died from the so-called "intestinal syndrome," which is comparable to that reported by Allen *et al.* (*8*).

Only 2 autologous animals had diarrhea or anorexia at any time after irradiation, and these conditions were transient and nondebilitating. There was no evidence of eye inflammation, mouth lesions, jaundice, or dermatitis, and the weight loss was not generally progressive; the original weight was quickly restored in all but 2 animals.

The clinical data demonstrate that no specific untoward reaction results from giving a monkey his own marrow after irradiation; the survival time in some of the autologous-treated animals now exceeds 1 year.

From observations in experiments with lower animals (*9*) it is apparent that 3 things may happen to homologous bone marrow after transplantation into an irradiated animal. Two of these situations were identified with certainty in the homologous-treated monkeys:

1) In some animals the marrow graft is apparently not accepted by the host, and death occurs after an interval comparable to that of an irradiated, nontreated animal. In the 3 monkeys showing no regeneration of hematopoiesis, the mean survival time was 15.3 days compared to 14.5 days for irradiated controls.

2) Even if the marrow graft is established in the host, it may later be destroyed by the host or in some other way rendered incapable of completely repopulating the peripheral blood with cellular elements. Such instances in rodents have been referred to as reversals. In many total reversals the host's own marrow regenerates to the point of maintaining the animal. It cannot be established with certainty whether this occurred in monkey #30. The alternate possibility is that it never accepted the grafted donor cells but regenerated its own hematopoietic system, which had temporarily been depressed by the sublethal radiation dose.

3) The last possibility is an establishment of the grafted bone marrow and the subsequent repopulation of the peripheral blood elements with donor-type cells. Ten monkeys showed this lasting take of the graft and probably maintained the donor-type cells until death. The mean survival time of these animals, 24.4 days, was significantly greater than in the controls.

The contrast between the results of the clinical observations in autologous- and homologous-treated monkeys indicates that the presence of foreign bone marrow can result in a number of severe reactions in the host animal. Fifteen of the 18 homologous-bone-marrow-treated animals suffered from severe diarrhea at some time after irradiation. The diarrhea was uncontrollable with the usual methods and antibiotic therapy had no effect on this condition; anorexia accompanied almost all diarrhea. These two conditions apparently accounted for a major portion of the weight loss in these animals. However, when optimal calories in a specially prepared diet were supplied by stomach-tube feedings, the weight loss was reduced but not halted.

The eye inflammation, mouth lesions, jaundice, and dermatitis that occurred in the homologous animals are another important point of contrast to the autologous animals.

Although these data are very suggestive of the occurrence of secondary disease in these animals, it is especially the autopsy data, as presented in (7), which demonstrate that many lesions found in the monkeys treated with homologous bone marrow are specific for secondary disease as defined in rodents.

The most striking result of this investigation is the demonstration of the short survival time of the monkeys which showed regeneration of hematopoiesis after homologous treatment, even when this regeneration was complete. This could be partly attributed to the general condition of the monkeys used, but cannot explain the marked difference between the autologous and homologous animals.

Death in the homologous animals seemed to be preceded by symptoms

of anorexia, diarrhea, wasting, and sometimes jaundice and dermatitis. In the light of the autopsy data these symptoms must be considered as part of the syndrome of secondary disease. Since these symptoms were apparent soon after irradiation (table 3), the short survival of these monkeys may be attributed to the early occurrence of secondary disease. In many animals the clinical syndrome was evident when there was not even convincing evidence of hematopoietic recovery.

The early occurrence of these symptoms is in many respects similar to the early and severe secondary disease seen in irradiated rodents after injection of mixtures of homologous bone marrow and lymphoid cells (*10*, *11*), spleen cells (*12–14*), or bone marrow mixed with blood (*15*, *16*).

In an attempt to elucidate the reasons for the similarity of effect of monkey bone marrow and rodent hematopoietic tissues with added lymphoid cells, the bone marrow cell suspensions injected into the monkeys were analyzed for cells from the lymphoid series. Usually the lymphoid cells were of the order of 25 percent in contradistinction to rodent bone marrow cell suspensions which contain approximately 10 percent lymphoid cells. It was calculated that the admixture of blood with the monkey bone marrow could be responsible for about ⅛ of the total number of lymphoid cells in the suspension, so that the bone marrow must have contained at least 15 percent lymphoid elements. This seems to support the hypothesis that the early occurrence as well as the severe nature of the secondary disease in the monkeys was due to the large number of lymphoid cells injected with the hematopoietic cells. This possibility has been discussed more extensively by one of us (*17*), in relation to the risks involved in foreign bone marrow transplantation.

However, a similarly severe secondary disease was reported in 2 children with leukemia who received after irradiation injections of a suspension of homologous adult bone marrow cells (*18*), though this suspension presumably did not contain a higher number of lymphoid cells than is usually encountered in rodents. Thus an alternative possibility might be that the severity of the syndrome is a peculiarity of the primate, both man and monkey, as distinct from the rodent.

Whatever the answer may be, it is evident that much additional information is required before clinical trials can be undertaken with confidence of success.

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Pathologic Changes in Irradiated Monkeys Treated With Bone Marrow [1,2]

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SUMMARY

Autopsy was performed on 27 irradiated monkeys. The 7 irradiated nontreated animals died of the bone marrow syndrome. Intestinal radiation death occurred in one treated with autologous bone marrow; bone marrow regeneration was delayed in the other animal that died with anemia and hemorrhagic pulmonary edema. In 3 animals treated with homologous bone marrow, there was no apparent regeneration—death was due to hemorrhage—but it occurred in the 14 others treated with homologous bone marrow cells. The pathologic changes closely resembled those found in mouse, rabbit, and human radiation chimeras suffering from secondary disease, which in the primate radiation chimera seems more severe than usually occurs in irradiated mice treated with foreign bone marrow. Since many lesions found in monkeys are more pronounced than those in rodents, this study has been illuminating in the pathogenesis of secondary disease. Cell loss, possibly a direct effect of an immunological reaction of the foreign bone marrow graft against the tissues of the host, was the primary lesion, amounting to severe necrosis and atrophy in many organs. In lesions of the intestinal tract, degenerative changes prevailed over regenerative while the reverse was true for early skin lesions. Although infectious disease occurred less frequently than in mouse chimeras (possibly because of treatment with antibiotics), impairment of the immunological defense system may have promoted invasion by commensal parasites or activation of latent infections. Mortality in animals treated with foreign bone marrow probably was caused by widespread denudation of ileum and colon and sometimes by massive liver necrosis or infectious disease.—J. Nat. Cancer Inst. 27: 67-97, 1961.

IN AN accompanying paper the clinical and hematological observations on groups of irradiated monkeys, treated with autologous or homologous bone marrow, are reported (*1*). The pathologic findings in the animals

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that died within 100 days after treatment are discussed and analyzed in the present paper. It will be shown that irradiated monkeys dying after a successful graft of foreign bone marrow was obtained show pathologic changes closely resembling those found in mice (*2–8*) and rabbits (*9*) suffering from secondary disease.

These observations in monkeys are of particular interest since similar changes have been reported in children with leukemia who died after total-body γ-irradiation and successful treatment with foreign bone marrow (*10*). It appears from these observations that the mortality of the primate radiation chimera may be explained by secondary disease as found in rodents.

MATERIALS AND METHODS

The data concerning the animals, irradiation, bone marrow suspensions, hematological examination, and treatment of irradiated monkeys have been reported in the preceding communication (*1*).

Autopsy usually was performed immediately after death. Femoral bone marrow and sometimes a rib were fixed in Bouin's fluid. The tissues were fixed in 4 percent formaldehyde solution and embedded in paraffin; sections were stained with hematoxylin and eosin. When required, special staining methods were employed: Mallory's phosphotungstic acid hematoxylin stain (PTAH) for fibrin, the periodic acid-Schiff (PAS) reaction, van Gieson's, crystal violet staining for metachromasia, and the performic acid-Schiff for keratin. Smears of femoral bone marrow were stained with May-Grünwald-Giemsa solution.

Usually bacteriological examinations of heart blood, spleen, liver, and intestinal contents were carried out.[8]

This communication reports the results of the examination of monkeys that died within 100 days after irradiation: 7 nontreated, 2 treated with autologous bone marrow, and 17 with homologous bone marrow. The data are summarized in table 1.

RESULTS

Incidence of pathologic changes in irradiated monkeys is shown in table 2.

Bone Marrow

Histological differences between nontreated monkeys irradiated with different doses were not apparent. The marrow was severely hypocellular (fig. 1). Between the dilated and congested sinusoids there were accumulations of plasma cells and histiocytes with ingested nuclear debris and erythrocytes. A few erythroblasts and myelocytes that usually showed pyknotic nuclei could be distinguished.

[8] The authors are indebted to Dr. F. Wensinck, Dr. A. A. van Soestbergen, and Dr. M. G. van Toorn for performing the bacteriological examination of the specimens.

Examination of the bone marrow revealed early regeneration in 1 of 2 monkeys treated with autologous bone marrow, and advanced regeneration in 13 of 17 monkeys treated with homologous bone marrow (table 2, fig. 2). Bone marrow regeneration occurred as early as 11 days post irradiation, after treatment with 47.8×10^8 homologous bone marrow cells (monkey #15, table 1).

A definite sequence of recovery was apparent when the cellular composition of the bone marrow specimens in the different animals was compared. The erythropoietic cell line appeared to recover first, followed by myelocytic and megakaryocytic recovery, in that order. After a stage of stem-cell hyperplasia, maturation seemed to occur. In some animals the matured bone marrow was hypercellular.

The sex chromatin data presented in the preceding paper (*1*) are in agreement with the histological findings of presence or absence of bone marrow recovery (table 2). In one animal classified as a take (T), only femoral bone marrow was examined, which proved to be aplastic. Since the peripheral blood counts showed a rise in neutrophil granulocytes, it seems likely that bone marrow regeneration occurred elsewhere. In 2 animals sex chromatin determinations were not practicable because of the small number of peripheral neutrophils. In these 2 monkeys (ID, table 2) histological examination showed bone marrow regeneration.

Hemorrhagic Diathesis

In all irradiated nontreated monkeys and in 3 treated with homologous bone marrow that failed to show regeneration, petechial hemorrhages had occurred in the skin, mucosal surfaces of the stomach, intestinal tract and bladder, serous membranes, myocardium, and adrenal gland. Extensive hemorrhage was in the muscles of the thigh at the site of previous injections, in the lung, particularly around foci of infestation with pneumonyssus, and in the cecum and ascending colon. In the latter localization, hemorrhage often resulted in necrosis of the entire intestinal wall (table 2), which led to perforation in 2 irradiated nontreated controls. Masses of bacteria were found in the necrotic tissue.

In the 13 animals treated with homologous bone marrow in which regeneration occurred, only 6 showed petechial hemorrhages at autopsy. In the majority of cases, the lymph nodes revealed that these animals had suffered transiently from hemorrhagic diathesis. The dilated sinusoids contained large numbers of erythrocytes and macrophages with ingested erythrocytes and iron pigment. Hemorrhagic necrosis of the colon did not occur, however. Monkey #51 died of extensive pulmonary hemorrhage; the lungs showed heavy infestation with pneumonyssus.

Septicemia

In most animals blood cultures made at autopsy showed some growth, mainly a few colonies of coliform bacteria. Furthermore, attempts to

TABLE 1.—Summary of findings and cause of death in irradiated nontreated monkeys and in those treated with autologous or homologous bone marrow

Treatment groups	Monkey No.	Sex	Treatment: X-ray dose (r)	Treatment: Bone marrow cells $\times 10^8$	Survival (days)	Clinical condition	Hematopoietic tissues	Infectious disease	Other lesions	Cause of death
Irradiated nontreated	31	♂	550		15	—	Bone marrow aplasia; atrophy lymphatic tissues	Septic necrosis	Hemorrhage; liver necrosis (septic)	Hemorrhage; septicemia
	39	♂	650		14	Mouth lesions	Bone marrow aplasia; atrophy lymphatic tissues	Septic necrosis; pneumonia; cystitis	Hemorrhage; liver necrosis (septic)	Hemorrhage; septicemia
	63	♂	650		15	Mouth lesions	Bone marrow aplasia; atrophy lymphatic tissues	Septic necrosis	Hemorrhage; liver necrosis (septic)	Hemorrhage; septicemia
	5	♀	700		16	—	Lymphatic tissue regeneration, presence of lymphatic follicles; bone marrow aplasia	—	Hemorrhage	Hemorrhage
	52	♂	810		15	—	Bone marrow aplasia; atrophy lymphatic tissues	Septic necrosis; oesophagostomiasis	Hemorrhage; liver necrosis (septic)	Hemorrhage; septicemia
	17	♂	910		15	Diarrhea	Bone marrow aplasia; atrophy lymphatic tissues	—	Hemorrhage; focal loss of crypt epithelium in colon and ileum; focal and zonal liver necrosis	Hemorrhage
	3	♀	1050		12	Diarrhea	Bone marrow aplasia; atrophy lymphatic tissues	—	Hemorrhage	Hemorrhage
Autologous bone marrow	12	♀	905	2.7	50	Edema	Early regeneration, hypocellular bone marrow; lymphatic tissues histologically normal	—	Hemorrhage; liver necrosis associated with severe fatty degeneration and anemia	Anemia; fatty degeneration of liver
	40	♀	1065	2.7	6	—	Bone marrow aplasia; atrophy lymphatic tissues	—	Focal loss of crypt epithelium in colon and ileum; denudation of duodenum and jejunum	Intestinal radiation
Homologous bone marrow	46	♂	600	15.2	19	Mouth lesions; jaundice; diarrhea	Early regeneration, hypocellular bone marrow; lymphatic tissue regeneration* and necrosis	Pericarditis	Crypt loss;† massive liver necrosis and dissociation; dermatitis	Intestinal denudation; liver necrosis
	57	♂	625	22.0	47	Edema	Bone marrow regeneration;‡ lymphatic tissue regeneration*	Pneumonia; yeast and virus infection	Crypt loss;† focal and zonal liver necrosis; dermatitis	Intestinal denudation
	28	♂	650	21.5	14	Jaundice; diarrhea	Bone marrow regeneration;§ lymphatic tissue regeneration* and necrosis	—	Crypt loss;† dermatitis	Intestinal denudation
	43	♂	650	24.8	31	Diarrhea	Bone marrow regeneration;‡ atrophy lymphatic tissues	Pneumonia; pericarditis	Crypt loss;† massive liver necrosis and dissociation; dermatitis	Intestinal denudation; liver necrosis; pneumonia
	27	♂	650	26.2	19	Jaundice; diarrhea	Bone marrow regeneration;§ lymphatic tissue necrosis; atrophy lymphatic tissues	—	Hemorrhage; crypt loss;† focal and zonal liver necrosis; dermatitis	Intestinal denudation

44	♂	650	37.4	10	Diarrhea	Atrophy lymphatic tissues; only femoral marrow examined; bone marrow recovery probably elsewhere (*I*)	Yeast infection	Crypt loss†	Intestinal denudation
15	♂	650	47.8	11	Diarrhea	Bone marrow regeneration;§ atrophy lymphatic tissues	—	Hemorrhage; crypt loss;† dermatitis	Intestinal denudation
51	♂	705	11.2	26	Edema; diarrhea	Bone marrow regeneration;§ lymphatic tissue regeneration, presence of lymphatic follicles	Oesophagostomiasis	Hemorrhage; focal loss of crypt epithelium in colon and ileum; dermatitis	Pulmonary hemorrhage
56	♂	740	24.0	23	Diarrhea	Bone marrow regeneration;§ lymphatic tissue regeneration*	Yeast infection	Hemorrhage; crypt loss;† massive liver necrosis and dissociation; dermatitis	Intestinal denudation; liver necrosis
54	♂	755	13.9	19	—	Bone marrow regeneration;‡ lymphatic tissue regeneration*	Pneumonia; oesophagostomiasis	Hemorrhage; crypt loss;† focal and zonal liver necrosis; not examined for dermatitis; nephrosis	Intestinal denudation; pneumonia
37	♂	795	7.9	17	Mouth lesions	Bone marrow aplasia; atrophy lymphatic tissues	—	Hemorrhage; not examined for dermatitis	Hemorrhage
53	♂	805	20.0	22	Mouth lesions; jaundice; diarrhea	Bone marrow regeneration;§ atrophy lymphatic tissues	Pneumonia	Crypt loss;† focal and zonal liver necrosis; nephrosis	Intestinal denudation
8	♂	855	8.0	13	Mouth lesions; diarrhea	Bone marrow aplasia; atrophy lymphatic tissues	—	Hemorrhage; focal and zonal liver necrosis; not examined for dermatitis	Hemorrhage
6	♂	865	8.5	27	Mouth lesions; edema; jaundice; diarrhea	Bone marrow regeneration;‡ atrophy lymphatic tissues	Virus infection	Focal loss of crypt epithelium in colon and ileum; focal and zonal liver necrosis; not examined for dermatitis	Ulcerative colitis associated with virus infection
32	♂	905	9.5	21	Mouth lesions; jaundice; diarrhea	Bone marrow regeneration;§ atrophy lymphatic tissues	Pneumonia; oesophagostomiasis	Hemorrhage; focal and zonal liver necrosis; not examined for dermatitis	Esophagostomum peritonitis
38	♂	915	13.7	14	Jaundice; diarrhea	Early regeneration, hypocellular bone marrow; atrophy lymphatic tissues	—	Focal loss of crypt epithelium in colon and ileum; focal and zonal liver necrosis; nephrosis; not examined for dermatitis	Hemolysis? Liver necrosis
41	♂	935	12.1	16	Mouth lesions; diarrhea	Bone marrow aplasia; atrophy lymphatic tissues	Oesophagostomiasis	Hemorrhage; focal loss of crypt epithelium in colon and ileum; not examined for dermatitis	Hemorrhage

*Early regeneration, groups of lymphoblasts, mitoses.
†Nearly complete loss of crypt and surface epithelium in colon and ileum.
‡Maturated bone marrow of normal or above normal cellularity.
§Cellular bone marrow with many stem cells.

TABLE 2.—Incidence of pathologic changes in irradiated monkeys

	Irradiated nontreated	Autologous bone marrow	Homologous bone marrow		
			T*	ID*	NT*
Bone marrow regeneration	0/7	1/2	11/12 †	2/2	0/3
Hemorrhagic necrosis of colon	6/7	0/2	0/12	0/2	2/3
Septicemia	4/7	0/2	0/12	0/2	0/3
Infectious disease‡	1/7	0/2	8/12	1/2	0/3
Intestinal denudation§	0/7	1/2‖	10/12	0/2	0/3
Extensive liver necrosis	0/7	0/2	3/12	0/2	0/3
Jaundice	0/7	0/2	5/12	2/2	0/3
Dermatitis	0/7	0/1	8/10	—	—
Lymphatic tissue regeneration	1/7	1/2	6/12	0/2	0/3
Lymphatic tissue necrosis	0/7	0/2	3/12	0/2	0/3

* Animals in which sex chromatin determinations indicated take or probable take (T) and no take (NT) of the graft, and animals in which a take could not be determined (ID) due to insufficient counts (*1*).

† In the negative animal only femoral bone marrow was examined; regeneration occurred according to the peripheral blood counts (*1*).

‡ Exclusive of septicemia and oesophagostomiasis.

§ Extensive loss of crypt and surface epithelium.

‖ Animal irradiated with 1065 r that died of typical intestinal radiation death on day 6.

culture microorganisms from the blood a few hours before death were always negative. Therefore, the diagnosis of septicemia was made only in monkeys that on histological examination showed necrotic foci with dense accumulations of microorganisms in the center, mainly in the liver. Cellular exudation was conspicuously absent.

Foci of septic necrosis were found in 4 of the 7 irradiated nontreated animals, but could not be detected in those treated with bone marrow.

Mouth

Ulcers of the oral mucosa were present in 2 irradiated nontreated monkeys. Microscopically the lesions consisted of complete necrosis of the mucous membrane which showed hemorrhage, infiltration with fibrin, and the presence of numerous bacteria in the vessels.

Lymphatic Tissues and Spleen

In 6 irradiated nontreated monkeys the lymphatic tissues of the lymph nodes, spleen, and intestinal tract were atrophied. Although lymphatic follicles were absent, a few lymphocytes were evenly dispersed between the reticular cells of the condensed connective-tissue stroma (fig. 3). The cortex of the lymph nodes was narrow. The sinusoids of lymph nodes and spleen were dilated, stuffed with erythrocytes, and often contained many histiocytes, some of which had ingested erythrocytes or iron pigment. Deposition of proteinaceous material frequently occurred in the remnants of the follicles in lymph nodes and spleen. This substance showed a negative reaction for fibrin with Mallory's PTAH stain.

One control monkey irradiated with 700 r showed advanced regeneration of the cortical tissue of the lymph nodes as evidenced by the formation of

lymphatic follicles. The medullary cords of the lymph nodes and the red pulp of the spleen were infiltrated with plasma cells.

In the animal treated with autologous bone marrow, which died 50 days after irradiation, complete regeneration of the lymphatic tissues with formation of follicles in the lymph nodes had occurred. In contrast the bone marrow showed only early regeneration.

Of the 17 animals treated with homologous bone marrow, 5 (autopsied on days 14, 19, 19, 23, and 47) showed early recovery of the lymphatic tissues, as indicated by the presence of varying numbers of lymphoblasts and mitoses (fig. 4). Another animal (day 26) showed advanced regeneration with many lymphocytes and formation of follicles in the cortical tissue of the lymph nodes, and many lymphocytes in the splenic white pulp.

In the lymph nodes of 3 animals, a peculiar necrosis was present (fig. 5). Extensive karyorrhexis of lymphoid cells had occurred, while the reticular stroma and the vessels were unaffected. Deposition of fibrinous substance was sometimes noted in the center of the follicles and medullary cords.

In the remaining 11 monkeys, autopsied on days 10, 11, 13, 14, 16, 17, 19, 21, 22, 27, and 31, there was atrophy of the lymphatic tissues, which usually was far more advanced than in the irradiated nontreated animals (fig. 6). The cortical tissue of the lymph nodes was extremely thinned and almost no lymphoid cells could be discovered. The deposition of proteinaceous substance with a negative reaction for fibrin in the follicles and collections of plasma cells in the medullary cords occurred regularly.

Liver

Septic necrosis of the liver has been mentioned earlier. This lesion was characterized by necrosis of hepatic parenchyma and supporting structures with no cellular exudation. One irradiated nontreated monkey showed unexplainable small foci of mainly pericentrally localized necrosis of parenchyma and stroma. Although the lesions histologically resembled septic necrosis, no microorganisms were seen.

Monkey #12, treated with autologous marrow and surviving 50 days, had extensive fatty infiltration of the liver with locally pericentral necrosis. These lesions can be explained by the severe anemia from which the animal suffered.

Of the 17 monkeys treated with homologous bone marrow, 12 revealed a type of liver necrosis which morphologically was clearly distinct from septic necrosis (table 2). In those livers that were only slightly affected the sections showed a diffuse sprinkling with necrotic liver cells as indicated by cytoplasmic eosinophilia, nuclear pyknosis, and karyolysis (fig. 7). Loss of liver parenchyma was also suggested by the occurrence of mitoses, which, probably as a result of irradiation damage, were often atypical.

In the more advanced cases the surface and cross section of the liver, on gross inspection, had a mottled aspect caused by multiple, pale-gray areas, which were not sharply demarcated. Microscopically, somewhat

extensive dissociation of liver-cell cords was present, which apparently often proceeded to focal, pericentral, or periportal necrosis (fig. 8). The intralobular connective-tissue stroma and the littoral cells characteristically appeared to be unaffected. The sinusoids and the peritrabecular lymphatic spaces were dilated and contained eosinophilic remnants of liver cells resembling Councilman bodies. The periportal connective tissue showed infiltration with mononuclear cells and increase of bile-duct structures. In addition, the epithelium of the smaller bile ducts showed swelling and increased eosinophilia of the cytoplasm, nuclear pyknosis, and occasional mitoses.

Jaundice

Of the 17 monkeys treated with foreign bone marrow, 7 showed jaundice at autopsy (table 2). This did not parallel the existence of liver necrosis, since 5 animals showed liver necrosis without jaundice and 1 suffered from jaundice without evidence of liver necrosis (table 1). In addition, the occurrence of jaundice was not clearly related to the extent of the liver lesions. Jaundice was not found in monkeys of the 2 other treatment groups.

Stomach and Intestinal Tract

Lesions caused by parasitic infections will be discussed in another section of this paper. Hemorrhagic necrosis of the colon, which led to perforation, has been described earlier.

In 1 of the 7 irradiated nontreated monkeys a few foci of cystic dilatation of mucosal crypts in the large intestine were found. In the center of these cystic spaces necrotic epithelial cells were desquamated.

In the autologous bone marrow group, one animal died 6 days after the irradiation (1065 r) in a state of severe dehydration. Histological examination of the intestines showed changes characteristic of intestinal irradiation death (*11*, *12*), with extensive denudation of the small intestine and crypt degeneration in the large intestine.

In most monkeys treated with foreign bone marrow, the mucous membrane of the lower ileum, large intestine, and occasionally the stomach were severely altered (table 2). The lesions explain the diarrhea from which most of the animals suffered (table 1).

In most cases intestinal dilatation and hyperemia of the mucous membrane were visible macroscopically, with unusual prominence of the remnants of the lymphatic follicles, which suggested mucosal atrophy. Sometimes the mucosal surface was covered with rather tightly adhering greenish-gray membranes. The duodenum and the jejunum were normal.

Microscopically the mucosa of the affected intestines showed necrosis of crypts, with desquamation of epithelial cells in the glandular lumina. Cystic dilatation occurred in the affected crypts, which were lined with flattened epithelial cells (figs. 9 and 10). The lamina propria and sub-

mucosa showed edema and infiltration with mononuclear cells, plasma cells, and occasionally neutrophil granulocytes. Areas of crypt degeneration alternated with those of a normal aspect (fig. 9) or areas in which apparently crypt regeneration had occurred as indicated by the lining of the glands with a hyperplastic epithelium that showed hyperchromatic nuclei, and the presence of many cell divisions (fig. 10).

In the more severely affected intestines, large stretches of the mucosa showed loss of surface epithelium and total disappearance of mucosal villi in the ileum and of crypts in the ileum and colon (fig. 11). The mucosal surface was covered with a membrane consisting of fibrin, desquamated epithelial cells, and disintegrated leukocytes. The lamina propria appeared to be thinned and showed condensation of the supporting connective-tissue stroma. In all these monkeys the jejunum and the duodenum were not at all or only slightly affected.

In 5 of the 17 monkeys treated with foreign bone marrow, only focal crypt degeneration could be detected, whereas in 10, extensive loss of crypt and surface epithelium had occurred (tables 1 and 2). Sex-chromatin determinations uniformly indicated a take of the graft in the latter animals. In the 2 in which the intestinal mucosa had a normal aspect (autopsied on days 13 and 17), bone marrow regeneration was absent.

Bacteriological examination of feces proved that 2 monkeys treated with homologous bone marrow harbored *Shigella flexneri* during the experiment. Histological examination did not reveal intestinal changes characteristic of dysentery, however. In the remaining 15, pathogenic microorganisms could not be cultured from feces or intestinal contents.

Kidneys

A tubular nephrosis resembling lower nephron nephrosis was found in 3 animals treated with homologous bone marrow. There were albuminous degeneration and focal necrosis of the convoluted tubules with epithelial desquamation. The tubular lumina were dilated and contained eosinophilic hyaline and granular casts, which, in the collecting tubules, were occasionally surrounded by fused desquamated epithelial elements, giving the appearance of giant cells.

Cardiovascular System

The myocardium of one animal treated with homologous bone marrow (#57, day 47) showed foci of atrophy and necrosis of muscle fibers. In the lesions an increase of fibroblasts and infiltration with histiocytes and mononuclear cells were observed.

Monkey #44, treated with homologous bone marrow, probably died of cardiac failure, because of the extreme congestion of the liver. Lesions of the myocardium could not be found.

Pericarditis occurred in 2 monkeys treated with homologous bone marrow. These lesions are described in the section on infectious diseases. Primary lesions of the vascular system were not seen.

Skin

Since only during the course of the studies we began to pay attention to macroscopically visible skin changes, examination of the skin was not carried out in the first 9 animals.

Histological changes of the skin were not seen in the 7 irradiated non-treated monkeys and in the 1 irradiated animal treated with autologous bone marrow whose skin was examined.

The majority of the animals treated with homologous bone marrow showed skin changes at autopsy. Macroscopically in a number of animals a macular erythema was apparent which centered in the skin of the face surrounding the orbits and mouth. In others the erythema extended to the skin of the anterior chest and abdomen. In addition, some showed extensive dry scaling of the skin of the ears and face (fig. 12). Edema of the subcutaneous tissues was present in a few animals.

Histologically the lesions in characteristic cases showed extensive acanthosis and hyperkeratosis, especially pronounced in the hair follicles, which led to great thickening of the follicular epithelium and plugging with keratin (fig. 13). In contrast, atrophy of hair follicles was noted elsewhere (fig. 14).

Foci of parakeratosis were sometimes found (fig. 14). In addition, large numbers of individually keratinized cells were present in the stratum spinosum and the hair matrix (fig. 15).

In one animal the superficial layers of the epidermis showed reticulating degeneration (fig. 14). Metaplastic changes of the epithelium were frequent in excretory ducts, probably of sweat glands, which were covered with concentrically layered squamous cells (fig. 16).

In the corium and subcutaneous tissues a variable amount of cellular exudation consisting of lymphoid cells and a small number of plasma cells and neutrophil granulocytes surrounded the hair follicles, sweat glands, and small vessels. Sometimes severe edema was present. In addition, an increase of fibroblasts was sometimes noted, which suggested early fibrosis.

The lesions varied from slight epidermal thickening with some plugging of follicles with keratin in some animals to excessive acanthosis, follicular hyperkeratosis, and dyskeratosis in others. In the 10 monkeys treated with homologous bone marrow from which skin specimens were examined, 8 showed these changes (table 2).

Testis

Since all the animals were of prepubertal age, spermatogenesis was absent in the testis. Histological changes were not apparent.

Infectious Disease

Helminths.—Infestation with *Œsophagostomum apiostomum* is of frequent occurrence in our colony. Since the lesions caused by this parasite

are well known (*13*), they will not be described here. One irradiated nontreated monkey and 4 irradiated monkeys treated with homologous bone marrow were infected to a varying extent. In animals #32 and 58, one of the colonic abscesses had perforated into the peritoneal cavity, which resulted in peritonitis.

In 2 other monkeys, eggs and larvae of *Strongyloides intestinalis* were found in the mucosal folds and crypts of the small intestine. An inflammatory response was absent.

Acariasis.—The majority of the monkeys from our colony were infected with pneumonyssus. On cross section, the lungs showed pinpoint cavities surrounded by a yellow margin. On microscopic examination the mite was found in cavities which resulted from dilatation of the smaller bronchi and which were surrounded by black-brown pigment. A mild, chronic, inflammatory response usually was apparent in the vicinity of the bronchiectatic lesions caused by the mite.

Sarcosporidiosis.—Striated muscles of 2 irradiated nontreated monkeys and 1 irradiated monkey treated with homologous bone marrow showed multiple localizations of *Sarcocystis kortei* (*13*). Inflammatory response in the surrounding tissue was absent.

Yeast infections.—Infection with a yeast, which formed pseudomycelia, was found in 3 monkeys treated with homologous bone marrow (table 1). Monkey #44 showed an ulcerative desquamating lesion of the facial skin. Histologically dermatitis and parakeratosis were seen, while numerous yeastlike organisms were interspersed between the keratin layers.

Monkey #56 suffered from necrotizing esophagitis. Yeasts and pseudomycelia were seen on the fibrin-covered surface of the mucous membrane and infiltrating into the necrotic tissue (fig. 17). Cultivation proved the organism to be *Candida albicans*.

Esophagitis with thickening of the epithelium, parakeratosis, and subepithelial abscesses, in which yeasts and pseudomycelia were present, were found in monkey #57.

Virus infections.—In 2 monkeys treated with homologous bone marrow, lesions suggestive of virus infection were found. Monkey #6 showed ulcerative colitis. Between the numerous neutrophil granulocytes, plasma cells, and mononuclear cells in the mucous membrane and submucosa, large cells with amphophilic oval nuclear inclusion bodies were noted (fig. 18). A number of these cells showed pyknosis, karyolysis, and disintegration of the cytoplasm. The giant cells and nuclear inclusions resembled those found in cytomegalic inclusion disease.

In monkey #57 lobular areas of the lung showed massive desquamation of alveolar cells and round-cell infiltration of the alveolar septa. A number of cells lining the alveoli and terminal bronchioles contained large, acidophilic, nuclear inclusions.

Miscellaneous inflammatory processes.—One irradiated nontreated monkey showed nonreactive bacterial pneumonia. The lesions consisted of necrosis of pulmonary tissue with fibrinous exudate and numerous bacteria in the alveoli. Five monkeys treated with homologous bone marrow had

bronchopneumonia (table 1). Slight cellular infiltration around foci of pneumonyssus was not classified as bronchopneumonia.

Cystitis was found in one irradiated nontreated monkey (table 1). Pericarditis of undetermined etiology occurred in 2 monkeys treated with homologous bone marrow. Both layers of the pericardium were covered with fibrin, thickened by edema and granulation tissue, and infiltrated with neutrophil granulocytes, plasma cells, and mononuclear cells.

Isolated Cellular Degeneration in Epithelial Tissues

In several organs, swelling of epithelial cells with increased cytoplasmic eosinophilia, nuclear pyknosis and the occurrence of mitoses were noted in a number of monkeys treated with homologous bone marrow. Apparent loss of individual cells as indicated by these changes occasionally was found in the liver, intrahepatic biliary ducts, adrenal cortex, pancreatic acini and islet tissue of the pancreas, salivary glands, and pelvis of the kidney.

DISCUSSION

The 7 irradiated nontreated monkeys died of the bone marrow syndrome. There were few, or no, hematopoietic cells in the bone marrow; nevertheless, the lymphatic tissues were not completely depleted of lymphoid cells, though lymphopoietic activity was not apparent. The liver lesions pointed to septicemia in 4 animals. Possibly the infection with *Œ. apiostomum* is a cause of septicemia in irradiated monkeys, since secondary bacterial infection of the lesions caused by the parasite is common (*13*). It is evident that apart from septicemia, hemorrhages contributed largely to the death of the animals.

Two points of interest have been presented. Lesions caused by pneumonyssus infection predisposed to extensive hemorrhage. Still more important was the occurrence of hemorrhagic necrosis of the cecum or colon, which might explain death either by perforation of the lesions into the peritoneal cavity or invasion by enteric microorganisms which resulted in septicemia.

One monkey treated with autologous bone marrow died 6 days after irradiation with 1065 r. The time interval and the autopsy findings were highly suggestive of intestinal irradiation death. The intestinal changes resembled those reported by Wilson (*12*). Contrary to the findings in mice that die of intestinal irradiation (*11*), the lesions of the large intestine were almost as severe as those found in the small intestine. For reasons not apparent, bone marrow regeneration was considerably delayed in the other animal that died, after treatment with autologous bone marrow, with anemia and hemorrhagic pulmonary edema on day 50. Histological examination of the bone marrow revealed only a few hematopoietic foci. This curiously contrasted with the nearly normal appearance of the lymphatic tissues. Regeneration of the lymphatic tissues also occurred in one of the irradiated nontreated animals.

The possibility of dissociation of lymphopoietic and hematopoietic recovery raises a point of practical importance. Conceivably, delay of treatment with foreign bone marrow beyond a critical time interval could result in failure of the graft to take, even after lethal X-ray doses. In one human case described by Mathé (#1, *10*) this might have been true.

Of the monkeys treated with homologous bone marrow, 3 died without convincing evidence of bone marrow regeneration, and their death could be explained by hemorrhage. Contrary to the observations in the untreated group, no evidence of septicemia was found.

The failure of the bone marrow treatment in these animals is probably related to the relatively small number of hematopoietic cells injected. An alternative explanation could be that the radiation dose applied was not sufficiently large to eliminate the immunological defense system of the host. An immunological reaction of the host against the graft might then be expected to prevent the take of the graft (*14*). This explanation seems unlikely, however, since treatment of all the other monkeys, even after irradiation with significantly smaller doses than applied in these 3 animals, resulted in bone marrow regeneration.

In 14 of the 17 monkeys treated with homologous bone marrow, death occurred in the presence of bone marrow regeneration. The observation that the recovery of megakaryocytes seems to occur later than recovery of myelo- and erythropoiesis may explain the findings that the majority of the animals have suffered from transient hemorrhagic diathesis. With the exception of one animal that died of pulmonary hemorrhage, however, neither septicemia nor hemorrage is likely to have contributed to the death of these animals.

Since bone marrow aplasia cannot account for the mortality, other explanations must be sought. It has been shown in mice that delayed mortality of radiation chimeras is related to an immunological reaction of the foreign bone marrow graft against the host (*14*). The mortality is preceded by clinical signs characteristic of so-called secondary disease.

Since in 11 of the 14 monkeys sex-chromatin determinations indicated a take of the homologous bone marrow graft (*1*), the mortality could have been caused by secondary disease. This assumption is strongly supported by the fact that the pathologic changes displayed by these monkeys closely resembled those reported to occur in mice (*2–8*) and rabbits (*9*) suffering from secondary disease: 1) necrosis and atrophy of the lymphatic tissues, 2) infectious disease, 3) massive liver necrosis, 4) dermatitis, and 5) loss of crypt and surface epithelium in the ileum and colon.

The changes of the lymphatic system in the monkeys presumably suffering from secondary disease seem to be crucial to the understanding of its pathogenesis. Although in the lymphatic tissues of irradiated mice treated with foreign bone marrow, lymphoid cells of the donor type can be demonstrated (*15*), histological examination usually shows atrophy without evidence of lymphopoietic activity (*6*). In hybrid mice treated with parental lymph-node cells in addition to bone marrow (*7*, *16*) or

with spleen cells (*8*), regeneration of the lymphatic tissues has been followed by degenerative changes of the lymphoid cells which ultimately resulted in atrophy. In all probability the destruction of lymphoid cells is related to an immunological reaction of the graft versus the host. The analogy of these changes to the destruction of lymphoid cells of the host in the process of rejection of a skin homograft and to sensitivity reactions of the delayed (tuberculin) type has recently been stressed (*7*, *17*).

In 6 of the 12 monkeys in which evidence of a take of the bone marrow graft was obtained, lymphopoietic activity occurred in lymph nodes and spleen. Necrosis of lymphatic tissue was found in 3 animals. The others showed atrophy of the lymphatic tissues. It has been stressed that the lymph nodes and the spleen follicles were more completely depleted of lymphoid cells than in the irradiated nontreated controls.

The enhanced susceptibility to infections in radiation chimeras has been related to the atrophy of the lymphatic tissues (*6*). The incidence of infectious disease in the monkeys treated with foreign bone marrow seems to be decidedly lower than in mouse chimeras. It seems probable that, in contrast to mice treated with foreign bone marrow, the majority of the monkeys more readily die of the primary effects of the graft-versus-host reaction.

Perforation of abscesses caused by *Œ. apiostomum* probably has contributed to the death of 2 animals. *S. flexneri* was cultured from the stools of 2 other animals. Since characteristic lesions were absent, it seems unlikely that dysentery contributed to the death of these monkeys.

It is difficult to evaluate pneumonia as a cause of death, since other important lesions were invariably present in the 5 animals concerned. The same applies to the yeast infections in 3 animals, infections of possibly viral etiology in 2, and occurrence of pericarditis in 2. Of interest, with respect to similar findings in human radiation chimeras (*10*), is the relatively frequent occurrence of an infection with a yeast which in one of the animals could be identified as *C. albicans*.

Since *C. albicans* is a normal inhabitant of the mouth, intestinal tract, and skin of monkeys (*13*), invasion of the organism could have been induced by the prolonged treatment with broad-spectrum antibiotics or the general impairment of the immunological defense system. The possibility of activation of latent infection in radiation chimeras is also suggested by the occurrence of lesions possibly virus-induced, since latent virus infections are widely prevalent in monkeys (*13*). Changes of a similar character have been reported in irradiated children treated with foreign bone marrow (*10*).

Although foci of liver necrosis occurred in 11 of the 17 monkeys treated with homologous bone marrow, massive necrosis and dissociation have been found in only 3 (table 2). Because of the resemblance of part of the lesions to the changes found in hepatitis, they might have been caused by virus infection or possibly activated latent virus infection. Focal necrosis of the liver in the other animals may as well have been caused by shock, anoxia, malnutrition, and allied conditions.

Seven animals treated with foreign bone marrow showed jaundice at autopsy. Since massive internal hemorrhage did not occur in these animals, and moreover, since neither the 7 irradiated nontreated controls nor the 7 animals treated with autologous bone marrow (*1*) exhibited jaundice, the irradiation probably is not an important factor in the production of this condition.

Furthermore, it has been shown that jaundice is not clearly related to morphological signs of liver damage. Jaundice may be due to hemolysis. Changes of the peripheral blood suggestive of hemolytic anemia have been reported in irradiated mice treated with foreign bone marrow (*6*). Hemolysis has also occurred in rabbit radiation chimeras (*18, 19*). The experiments of Porter (*19*) indicate that the hemolysis in rabbit chimeras is due to destruction of host-type erythrocytes. The phenomenon is therefore compatible with a graft-versus-host reaction. If hemolysis occurred, it probably did not contribute significantly to the mortality of the monkeys. The only lesion that might be attributed to hemolysis is the tubular nephrosis, found in 2 jaundiced animals. In only 1 of these, no lesions could be found to account for the death of the animal other than tubular nephrosis and diffuse, isolated degeneration of liver cells.

The histological changes of the skin reported here have not been described earlier in irradiated animals treated with bone marrow. Atrophy of the epidermis and fibrosis of the cutis, probably the final stage of the dermatosis, have been reported to occur in mice (*6*). The skin lesions of the monkeys are distinguished from those of the liver and the intestinal tract by the prevalence of proliferative changes (acanthosis and metaplasia of the excretory ducts of the sweat glands) over degenerative changes (dyskeratosis, parakeratosis, reticulating degeneration, and atrophy of hair follicles).

It appears that the cell loss is morphologically represented by the individual keratinization of the cells of the epidermis and hair matrix. In contrast to the regenerative changes similarly occurring in the intestinal lesions, proliferation of the basal layers of epidermis and hair follicles compensates amply for the cell loss in the early stage. Some histological features of the dermatosis seem to be characteristic of homotransplantation reactions. Similar changes have been described in sex-incompatible skin grafts in C57BL mice (*20*), long-term skin-graft rejection in mice (*21*) and hamsters (*22*), and rats suffering from runt disease (*23*). In the former 2 the graft rejection takes a prolonged course. Furthermore, the inflammatory response is predominantly localized around the hair follicles, also a feature of the dermatosis in monkeys.

Because of the extensive lesions of the intestinal tract exhibited by 11 of the 17 monkeys treated with foreign bone marrow, intestinal irradiation damage must be considered in the explanation of the death of the animals. Several observations render this unlikely.

Only 1 of the irradiated nontreated monkeys (910 r) showed slight focal crypt degeneration in the large intestine, not comparable to the extensive denudation in monkeys treated with foreign bone marrow. This is

corroborated by the observation of Wilson (*12*) that monkeys irradiated with 900 r from a 250 kv Picker therapy machine only occasionally show focal epithelial lesions of the intestine. Secondly, no parallelism exists between the extent of the lesions and the radiation dose administered (table 1). Similarly, no parallelism exists between the extent of the lesions and the time interval between irradiation and death. The latter observation is important with respect to the finding of Wilson (*12*) that the distribution of the colonic lesions is a function of survival time rather than of dose over the dose range tested (1500–7500 r). The time interval between the irradiation and mortality in monkeys dying of intestinal radiation has been found to be 5 to 9 days (*12*). This is supported by our observations in monkey #40, which died 6 days after irradiation with 1065 r. The animals treated with foreign bone marrow lived considerably longer than the stated interval. Finally, in monkey #40, treated with autologous bone marrow, which died 50 days after the irradiation, intestinal lesions were notably absent.

Although it is improbable that the monkeys have died of intestinal irradiation death, it cannot be excluded that irradiation-induced damage has been an additional factor in the pathogenesis of the intestinal lesions (*14*). The extent of the lesions may readily explain the death of the animals by disturbance of electrolyte metabolism, loss of protein, or both. This is supported by the experiment of Friedberg (*24*), who showed that loss of serum-albumin occurred in irradiated mice treated with foreign bone marrow. A state of shock caused by serum-albumin loss might result in tubular lesions of the kidney found in 3 animals.

Lesions of the intestinal tract differ in one important aspect from those in irradiated mice treated with foreign bone marrow. Inflammatory changes prevailed in the mice (*6*), while in the monkeys cellular exudation was of minor significance. This difference is highly elucidating with respect to the pathogenesis of the intestinal changes. In mice it has not been possible to decide between primary bacterial infection or secondary infection of pre-existent lesions.

Although the absence of serious inflammation in the monkey might have been caused artificially by treatment with antibiotics, the observations strongly suggest that the destruction of intestinal epithelium is the primary lesion and occurs independently from bacterial infections. It may be postulated, therefore, that the destruction of crypt epithelium is not related to the decreased resistance to bacterial infection but results directly from the graft-versus-host reaction.

If it is assumed that the epithelial cells of the intestinal crypts are a target for the immunological activity of the graft, the intestinal denudation may be explained either by crypt necrosis or by inhibition of crypt-cell proliferation necessary to compensate for the normally occurring cell death in the surface epithelium and the irradiation-induced damage.

Similar reasoning may be applied to the necrosis and the individual loss of cells occurring in the liver, skin, and, occasionally, myocardium, kidney, and epithelial tissues of a diversity of organs. Probably significant with

respect to the pathogenesis of these lesions is the constant slight lymphoid-cell infiltration.

Finally, it remains to be investigated why the secondary disease in monkeys seems to be much more severe than usually is described in other animal species. In fact, the mortality pattern of the monkeys treated with foreign bone marrow is comparable to that of mice treated with foreign lymphoid cells in addition to bone marrow (*16*): Survival does not occur and the animals die earlier than mice treated only with foreign bone marrow. As was pointed out in the preceding paper, the simplest explanation is provided by the assumption that the monkey bone marrow suspensions contain a relatively higher number of lymphoid cells.

It seems of prime importance that identical lesions have been described to occur in irradiated children treated with homologous bone marrow (*10*). The striking similarity of the histology and severity of secondary disease between these human cases and the data described in this communication justify extensive further study of bone marrow transplantation in the monkey.

ADDENDUM

After the completion of the manuscript, an additional 9 monkeys were irradiated with 900 r and treated with *autologous bone marrow.* (This work was done in collaboration with Dr. E. Magliulo.)

The animals were serially killed, 1, 2, 3, and 4 weeks after the irradiation (2, 3, 2, and 2 animals each time, respectively).

Bone marrow recovery occurred to a varying extent in 8 of these monkeys. In 6 evidence of regeneration of lymphatic tissue was found. Denudation of the intestines, liver necrosis, jaundice, dermatitis, necrosis of lymphatic tissue, and isolated cell degeneration were notably absent in these animals.

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PLATES

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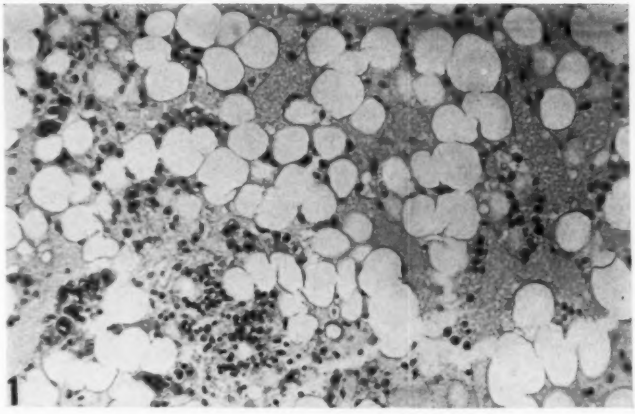
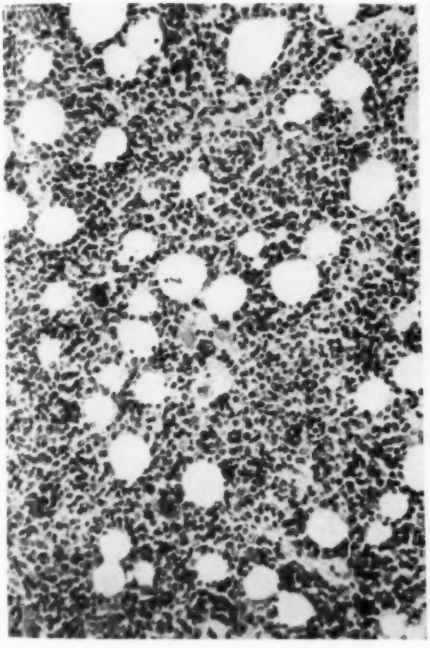
Plate 8

Figure 1.—Bone marrow aplasia. Few megakaryocytes, pyknotic erythroblasts, and collections of plasma cells and histiocytes are present among reticular cells. Nontreated monkey #17 that died 15 days after total-body irradiation (910 r). Hematoxylin and eosin. × 192

Figure 2.—Advanced regeneration of bone marrow with stem-cell hyperplasia and relatively few mature cells of myeloid series. Monkey #51 that died 26 days after total-body irradiation (705 r) and treatment with homologous bone marrow 11.2×10^8 cells. Hematoxylin and eosin. × 192

Figure 3.—Atrophy of cortical tissue of lymph node. Follicles absent, but a few lymphocytes dispersed in reticular stroma. Nontreated monkey #52 that died 15 days after total-body irradiation (810 r). Hematoxylin and eosin. × 192





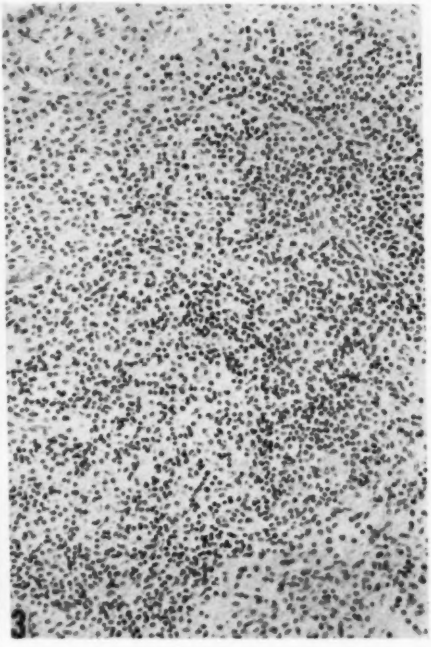


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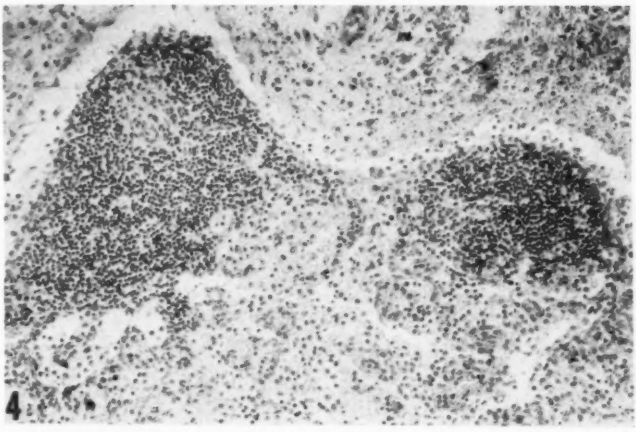
PLATE 9

FIGURE 4.—Lymph node showing focal regeneration of lymphatic tissue, with early formation of follicle. At *top* many pyknotic and fragmented lymphoid cells are present. Monkey #28 that died 14 days after total-body irradiation (650 r) and treatment with homologous bone marrow 21.5×10^8 cells. Hematoxylin and eosin. × 120

FIGURE 5.—Same lymph node as shown in figure 4. Necrosis of lymphatic tissue. Many pyknotic and fragmented lymphoid cells dispersed in unaffected reticular stroma. Hematoxylin and eosin. × 120

FIGURE 6.—Extreme atrophy of cortical tissue of lymph node. Sinusoids dilated and filled with histiocytes, some of which have ingested erythrocytes. Monkey #38 that died 14 days after total-body irradiation (915 r) and treatment with homologous bone marrow 13.7×10^8 cells. Hematoxylin and eosin. × 192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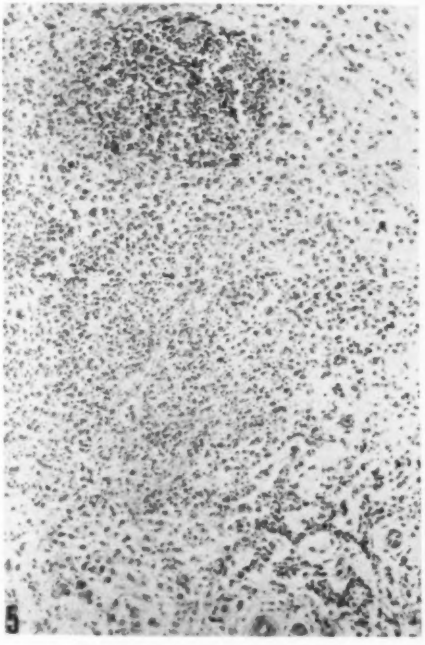
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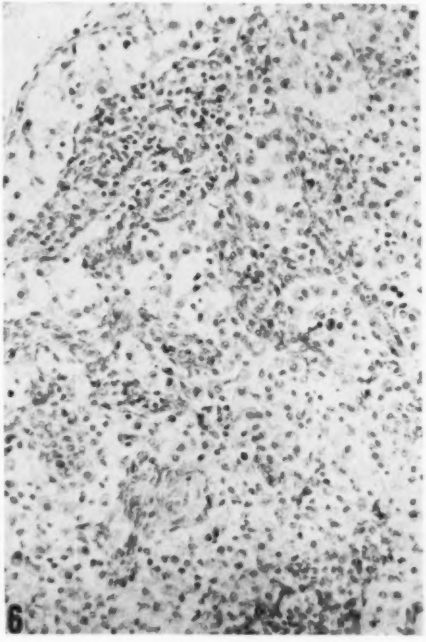
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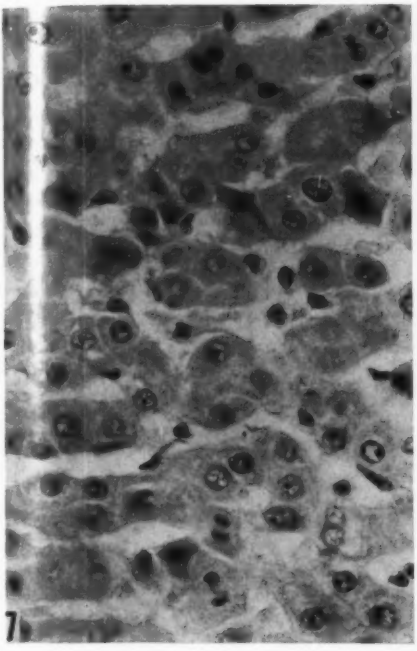
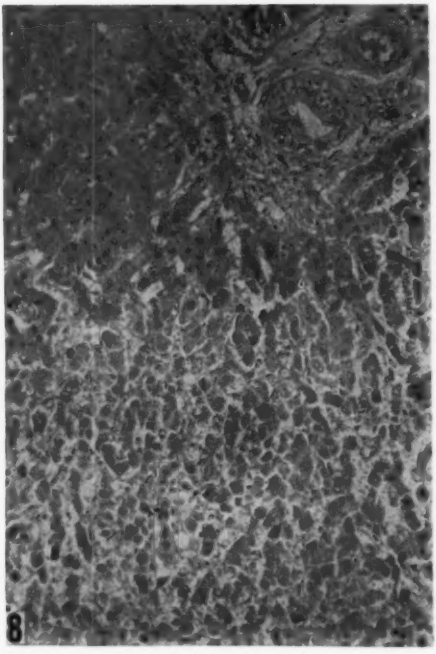
PLATE 10

FIGURE 7.—Liver showing degeneration of individual parenchymal cells and mitoses. Same monkey as in figure 6. Hematoxylin and eosin. × 480

FIGURE 8.—Massive dissociation and necrosis of hepatic parenchyma. Monkey #56 that died 23 days after total-body irradiation (740 r) and treatment with homologous bone marrow 24.0 × 10^8 cells. Hematoxylin and eosin. × 120

FIGURE 9.—Loss of crypt and surface epithelium in ileum. Area of mucosal atrophy borders on area with only minor changes. Monkey #54 that died 19 days after total-body irradiation (755 r) and treatment with homologous bone marrow 13.9 × 10^8 cells. Hematoxylin and eosin. × 30





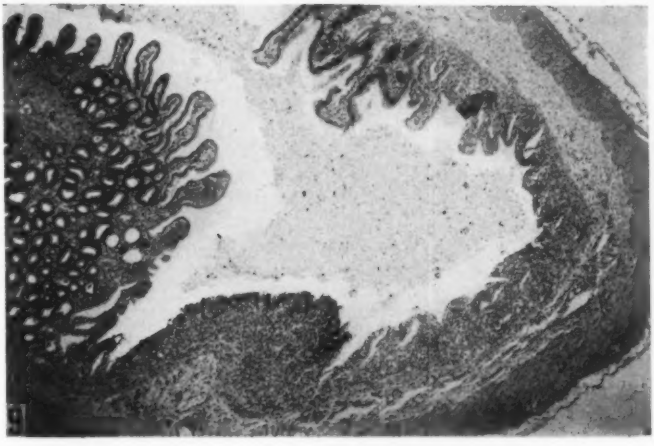


PLATE 11

FIGURE 10.—Colon showing cystic crypt degeneration and a few regenerating hyperplastic crypts. Lamina propria shows slight round cell infiltration. Same monkey as in figure 2. Hematoxylin and eosin. × 120

FIGURE 11.—Total loss of crypt and surface epithelium in colon. Mucosal surface covered with layer of fibrin and neutrophil granulocytes. Same monkey as in figure 4. Hematoxylin and eosin. × 120

FIGURE 12.—Dermatitis with scaling of ear and skin of face. Monkey #57 that died 47 days after total-body irradiation (625 r) and treatment with homologous bone marrow 22.0 × 10^8 cells.

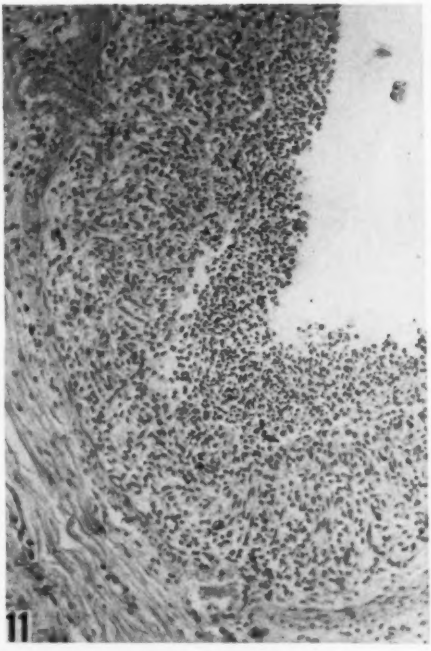
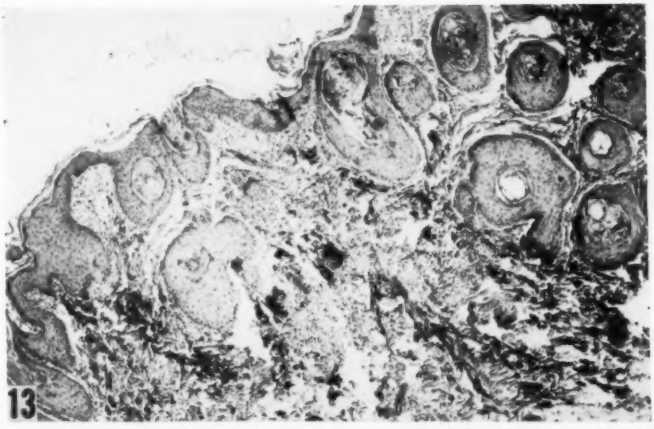


PLATE 12

FIGURE 13.—Acanthosis and follicular hyperkeratosis of skin of face. Same monkey as in figure 2. Hematoxylin and eosin. × 30

FIGURE 14.—Skin of face showing acanthosis, parakeratosis, dyskeratosis, and reticulating degeneration of upper epidermis. Several atrophic hair follicles present. Round-cell infiltration around follicles and small vessel. Monkey #46 that died 19 days after total-body irradiation (600 r) and treatment with homologous bone marrow 15.2×10^8 cells. Hematoxylin and eosin. × 120

FIGURE 15.—Collection of dyskeratotic cells in basal layer of epidermis. Same monkey as that in figure 14. Hematoxylin and eosin. × 480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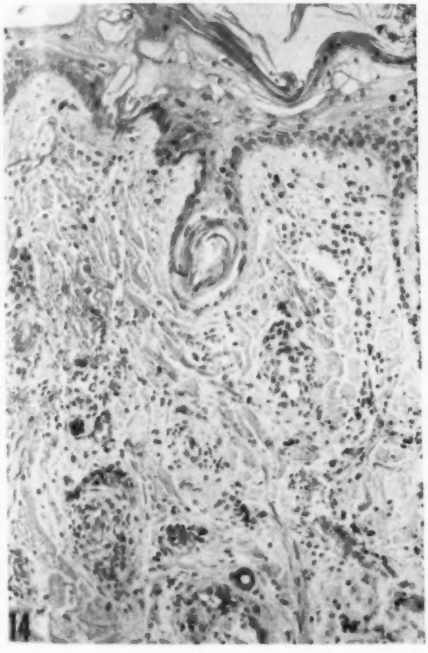
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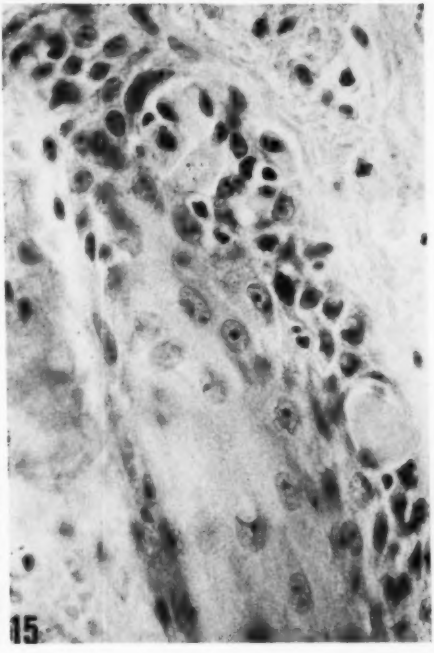
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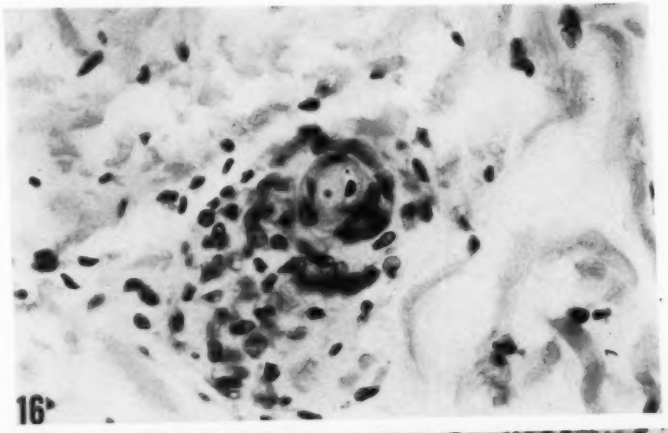
PLATE 13

FIGURE 16.—Squamous metaplasia of excretory duct, probably of sweat gland. Skin of face of monkey #51, shown in figure 2. Hematoxylin and eosin. × 480

FIGURE 17.—Necrotizing esophagitis with invasion of mucous membrane by yeast-forming pseudomycelia. Same monkey as in figure 8. Hematoxylin and eosin. × 480

FIGURE 18.—Ulcerative inflammation of colonic mucosa with many giant cells showing amphophilic nuclear inclusion bodies. Monkey #6 that died 27 days after total-body irradiation (865 r) and treatment with homologous bone marrow 8.5×10^8 cells. Hematoxylin and eosin. × 480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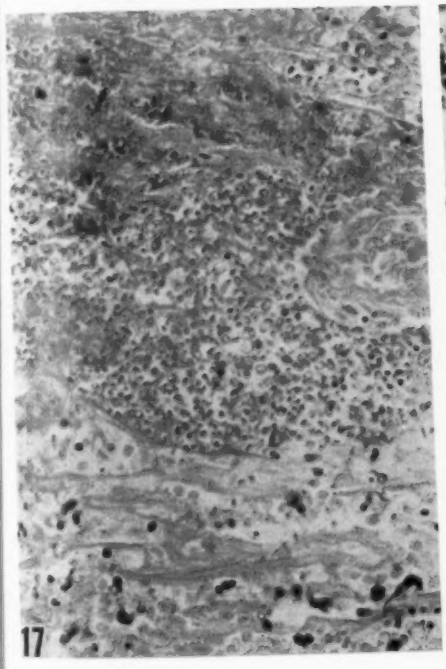
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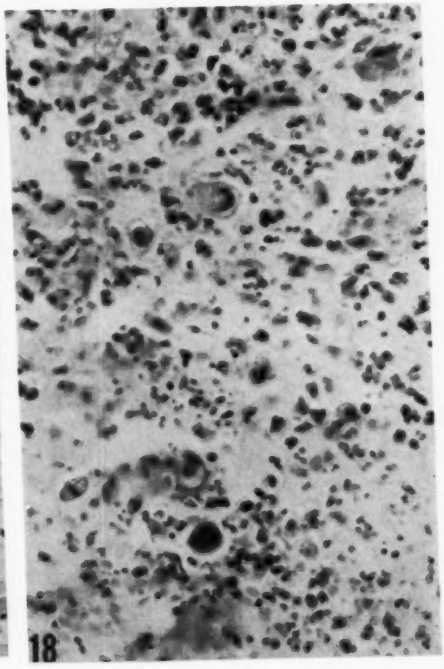
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93953—61——12

Study of Host-Virus Relationship. I. Light, Phase, and Fluorescence Microscopy of Mouse-Embryo Cells Infected With Polyoma Virus [1, 2]

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SUMMARY

Comparative studies by means of light, phase-contrast, and fluorescence microscopy were carried out on C3H/An/Dm mouse-embryo cell cultures infected with SE polyoma virus. The nature and sequence of changes after infection were studied. As seen by phase microscopy, inclusions appear in the nuclei of the infected cells, 4 to 5 days after infection. One type of inclusion, centrally or eccentrically located, is composed of dense, homogeneous material and is frequently surrounded by a clear zone. The other type of inclusion consists of either dense strands or amorphous, dense, scattered material. Increase and margination of chromatin material are frequently observed. The inclusions increase in size and density before the lysis of cells. Both types of inclusions stain yellow for deoxyribonucleic acid (DNA) with acridine orange fluorescent dye. The staining is not removed by treatment with deoxyribonuclease (DNase) or pepsin. Pretreatment with pepsin, followed by DNase digestion, removes the DNA stain, which indicates the possible viral nature of these inclusions. By the Feulgen method, both types of inclusions at first stain delicate pink and lavender-pink at the height of infection. Feulgen-negative material surrounds the dense areas of type-1 inclusions and is present between the dense strands or amorphous material of type-2 inclusions. During the last stages of infection, inclusions of similar staining properties are found occasionally in the cytoplasm of the infected cells. Electron microscopy of ultrathin sections of similar cells or adjacent thin sections of cells with the two types of intranuclear inclusions has revealed characteristic polyoma-virus particles within these inclusions. The chemical nature of the polyoma virus is discussed in the light of these observations.—J. Nat. Cancer Inst. 27: 99–129, 1961.

STEWART AND her associates isolated from leukemic tissues of mice an agent described as polyoma virus, which induces multiple tumors in animals of several species and a cytopathic effect in mouse-embryo cells grown in tissue culture (*1–3*). Cytochemical studies on changes induced by this agent in cells of a milk-adapted murine lymphoma strain have

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[3] U.S. Public Health Service Predoctoral Fellow.

[4] The authors gratefully acknowledge the excellent help of Mr. Frank Padgett in preparation of the photographs.

593953—61——13

been reported by Love and Rabson (*4*). Henle and her associates (*5*) described the changes induced by the polyoma virus in mouse-embryo cells by using fluorescent antibody staining technique. Malmgren and his associates (*6*) studied by means of fluorescein-labeled antibodies the behavior of polyoma virus in cells of a murine lymphoma (strain P388 D_1) grown *in vitro*.

In connection with these observations, studies were undertaken on the nature and sequence of changes in susceptible cells after infection with polyoma virus. These studies were carried out by phase, fluorescence, and electron microscopy combined with light microscopy of cytochemically stained mouse-embryo cells infected with the polyoma virus.

In this paper, the changes observed chiefly by light, phase, and fluorescence microscopy will be presented, and in (*7*) changes observed by electron microscopy will be described.

MATERIALS AND METHODS

Cell cultures.—As a source of material for tissue cultures, embryos from Bittner agent-free C3H/An/Dm mice were used. The embryos were removed under sterile precaution, minced, washed with Eagle's balanced salt solution (BSS) and trypsinized for 35 minutes with a 0.025 percent solution of Bacto-trypsin (Difco Laboratories, Detroit, Mich.). The cells were centrifuged for 15 minutes at 900 $\times$ *g*, the supernatant containing the trypsin was removed, and the sediment containing the cells resuspended in Eagle's BSS with 20 percent calf serum. Approximately 1×10^6 cells in 10 ml of medium were inoculated into T-30 flasks and incubated at 37° C. After the mouse-embryo cells formed monolayers, usually within 3 to 5 days, they were trypsinized, resuspended in the complete medium, and approximately 10^5 cells inoculated into special tissue-culture chambers (*8*). After a cell monolayer was obtained, usually within 24 to 48 hours, the medium was replaced with Eagle's BSS with 2 percent horse serum. After change of the medium, SE-polyoma-virus preparations were inoculated into the tissue-culture chambers. The medium was replaced by fresh medium as soon as acidity in the fluid was noted.

Several strains of polyoma virus (3343, 269, TR6, 11258, and PTR5189), originally obtained through the courtesy of Dr. Sarah Stewart and carried in our laboratory, were used as a source of virus in the present experiments. All strains induced similar characteristic changes in mouse-embryo cultures, though the time of appearance of the changes varied somewhat from strain to strain of the polyoma virus.

The stock suspension of virus for experiments was prepared by inoculation of C3H/An/Dm mouse-embryo cell monolayers in T-30 flasks with 1 ml of the original virus preparation. The medium containing 2 percent horse serum was usually changed after 7 days, and the tissue-culture fluid saved when cytopathic changes were observed. This usually occurred on the 11th to 14th day after virus inoculation. The tissue-culture material

was stored at −70° C, unless used for immediate passage in tissue-culture chambers.

The stock suspensions of virus were titrated in T-30 tissue-culture flasks containing C3H/An/Dm mouse-embryo cell monolayers by the addition of 1 ml of the suspension to 9 ml of Eagle's BSS and 2 percent horse serum. Serial tenfold dilutions of the stock suspension of virus were used. At least 4 flasks were used for each dilution. Control flasks were inoculated with uninfected medium. The cultures were observed daily for cytopathic effect.

For experiments, stock suspensions of virus showing titer of tissue-culture infectious doses (TCID50) of 10^{-5} to 10^{-6} were used. The virus was inoculated in 0.2 ml quantities into the tissue-culture chambers that held a total of 0.7 ml fluid and contained approximately 10^5 cells at the time of inoculation.

Cytological techniques.—The chambers were examined daily by the phase microscope and phase photomicrographs were taken regularly as changes occurred. Studies were made on cells from several chambers taken at daily intervals after infection. Coverslips from the chambers were stained by the Feulgen method for light microscopy and by acridine orange for fluorescence microscopy (*9*).

For Feulgen staining, the cells were fixed for 10 minutes in Carnoy's fluid (6 parts 95% alcohol, 3 parts 10% formalin, and 1 part glacial acetic acid); the hydrolysis time in 1 N HCl acid was 25 minutes at 56° C and staining with Schiff's reagent was carried out overnight. This modification of the Feulgen procedure was adapted after it was observed that tissue-culture preparations of control untreated mouse-embryo cells did not stain after the usually prescribed ½ to 1 hour in the Schiff reagent. A dilute (5%) solution of light green in 95 percent ethyl alcohol was used for counterstaining.

Prior to staining with acridine orange for examination with the fluorescence microscope, the coverslips were fixed in Carnoy's fluid as for the Feulgen stain, washed in McIlvain's buffer (*p*H 4.0), and stained for 20 minutes in a 1:10,000 concentration of acridine orange at *p*H 4.0, washed again with fresh buffer, and mounted wet. The Carl Zeiss WL microscope stand was used, with illumination provided by an Osram HB0200 mercury lamp and appropriate exciter and barrier filters. Photographs of preparations stained with acridine orange were made on 35 mm Super-Anscochrome film, the exposures varying from 1 to 5 seconds, the usual exposure being 2 seconds.

Nucleic acid digestion, prior to staining with acridine orange, was carried out by the use of deoxyribonuclease (DNase), 2 × crystallized, obtained from Worthington Biochemical Corporation, Freehold, New Jersey, at a concentration of 200 μg per ml of $MgSO_4$ solution at *p*H 7.0 for 1 hour at 37° C. For protein digestion, pepsin was used at a concentration of 200 μg per ml of distilled water at *p*H 2.0 for 1 hour at 37° C, either alone or followed by DNase treatment, prior to staining with acridine orange.

Electron microscopy.—Coverslips with monolayers of mouse-embryo cells infected with polyoma virus, selected for electron microscopy, were washed 3 times (5 minutes each) in Tyrode's solution, then fixed for 20 minutes in a 1 percent solution of Veronal-acetate-buffered osmic acid at *p*H 7.4, containing 0.045 gm of sucrose per ml of fixative. The cells were then washed for 20 minutes in Tyrode's solution, dehydrated in 70 percent alcohol containing 3 percent uranyl nitrate for 20 minutes, and subsequently in 95 percent and 100 percent for 10 minutes each. The cells were then treated with a mixture containing equal parts of 100 percent alcohol and butyl methacrylate for 20 minutes followed by butyl methacrylate. For embedding in butyl methacrylate, the cells were scraped from the coverslip with a rubber policeman, spun at 1500 $\times$ *g* for 10 minutes, and kept at 48° C overnight for polymerization.

Bioassays.—Biological tests were carried out with tissue-culture fluids from cultures in which cells showed the characteristic changes to be described. Hamsters, 2 to 3 days old, were used as test animals.

RESULTS

Phase Microscopy

No characteristic changes have been observed in the mouse-embryo cells for 2 to 3 days after inoculation with the SE polyoma virus. Two or 3 days after infection, nucleoli of some cells become bizarre-shaped, coarse, or enlarged (figs. 1, 2, and 3). Small, dark granules, gradually increasing in number, appear throughout the nucleus (fig. 2). At this stage of infection, the nucleus is occasionally filled with dark material, as shown in figure 4. Some cells show vacuoles in the cytoplasm filled with material of lesser density than the surrounding cytoplasm. In some vacuoles dark granules can be observed (fig. 5). The changes observed in the nuclei of cells in the infected cultures have not been seen in the control cultures. Active cell division has been observed in both control and inoculated cultures. Cells in both types of cultures sometimes show increased vacuolization and granularity of the cytoplasm (figs. 5 and 6).

Inclusions appear in the nuclei of some cells on the 4th or 5th day and occasionally on the 3d day after infection. These inclusions are found centrally or eccentrically located in the nucleus. They gradually increase in size, occasionally almost completely filling the nucleus. The appearance and location of the inclusions are shown in figures 7 through 12. The dark inclusion material fills most of the nucleus, as shown in figure 7, and surrounds the nucleolus (fig. 8). The cytoplasm shows vacuolization and granulation (fig. 8). The dense homogeneous nuclear inclusions may be surrounded by a halo and the nuclear membrane is frequently sharply outlined (fig. 9). The inclusions, one or more, may also appear as gray bodies surrounded by a halo (fig. 10). Chromatin material frequently may be seen aggregated around the nuclear membrane (fig. 10).

The nuclear inclusions sometimes assume other shapes, as shown in figures 11 and 12. Apparently uninfected cells are seen in the vicinity of the infected cells (fig. 12). Because of the characteristic appearance of these inclusions, they will be referred to as type-1 inclusions.

Along with the development of this type of inclusion, cells in mitosis and inclusions of a different appearance (to be described) have been observed in the infected cells. No gross cytopathic changes, as described by Stewart (*2*), could be seen at this stage of infection. Cells that contain type-1 inclusions may live for 1 or 2 days, and then die after lysis of the nucleus. Cells containing inclusions have at no time as yet been seen to divide. (Bereczky and Dmochowski: Data from time-lapse photography. To be published.)

In a large number of infected cells, scattered strands of gray material of varying density have been observed in the nuclei. They fill the nucleoplasm (fig. 13) or may occasionally be seen in association with the nucleoli (fig. 14). This material gradually becomes more widespread and dense (figs. 15 through 18). Some dark material frequently appears at the periphery of the nuclear membrane (figs. 14 and 15). The nucleolus may be surrounded by a halo (fig. 15) and occasionally may become vacuolated (fig. 16). In cells with this type of inclusion, the cytoplasm may become extremely granulated (figs. 16 and 17). Enlarged nuclei and binucleated cells showing this particular reaction to infection with the polyoma virus, described as type-2 inclusion, have frequently been observed. No connection could be found between type-1 inclusions and type-2 inclusions. Type-1 inclusions appear occasionally throughout the whole period of infection, while type-2 appear consistently in large numbers. Binucleated cells are usually infected with the same type of inclusions. However, neighboring cells sometimes may contain a response of either type (fig. 19).

Six to 9 days after infection with the polyoma virus, the majority of the cells appear infected, with many clear areas in the culture where the cells have lysed. Patches of destroyed cells can be seen throughout the culture; the nuclei are pyknotic, and the cytoplasm sometimes contains dark, spherical inclusions. These cytoplasmic inclusions may appear to be pyknotic nuclei, but they contain characteristic virus particles, as shown by electron-microscope studies. These cytoplasmic inclusions will be described in detail in the second paper (*7*) on the basis of staining with acridine orange and the appearance in the electron microscope. The rest of the cytoplasm is dense and granular (fig. 16). Type-2 nuclear inclusions increase in size through accumulation of the amorphous material (fig. 17). The dense strands of gray material become thickened, with a light-gray material appearing between the strands of the network. The cytoplasm appears filled with small, dark granules and vacuoles (fig. 17).

In control cultures, 9 days old or older, dead cells are more frequently found, but nuclei of living cells do not show any changes. There may be some increase in granularity of the cytoplasm, but as a whole the cytoplasm is distinct and clear (figs. 21 and 22).

In the 9-day-old or older infected cultures, the individual cells show more prominently type-2 inclusions. The nucleus may appear considerably darker at this stage of infection (fig. 18). A nucleus completely filled with the very dark, amorphous material is shown. Other such nuclei may be opaque and glistening. Many clear areas of lysis are evident. There is a massive cell death, condensation of the nucleus, shrinking of the cytoplasm, and finally the whole culture assumes a grapevine appearance characteristic of the cytopathic effect (fig. 20).

Fluorescence Microscopy

During the first 2 or 3 days after infection, no difference can be observed between cells from the inoculated and control cultures stained with acridine orange fluorescent dye. Two or 3 days after infection, enlargement and increased ribonucleic acid (RNA) fluorescence of the nucleolus are the most prominent features. This indicates that the nucleolus may play an important part in the multiplication of polyoma virus. However, on the 3d or 4th day, type-1 inclusions appear within the nucleus and stain bright yellow. As indicated by the yellow, specific for deoxyribonucleic acid (DNA) (*9*), the DNA-containing material is found in the inclusions, in marginated chromatin, and in strands within the nucleus (figs. 23 through 28). The cytoplasm remains red, indicative of RNA, except for small bodies which stain yellow or yellowish-green for DNA (figs. 23 and 24).

The amorphous material of type-2 intranuclear inclusions also stains as DNA with acridine orange, while the nucleolus material stains red or red-orange (fig. 25). The distribution of DNA-staining material around the nuclear membrane may be seen in figure 26. Apparently healthy cells are frequently in the vicinity of the infected cells. Infected nuclei of cells during the later stages of infection (6 to 9 days or over) also reveal amorphous material staining as DNA (figs. 27 and 28). Different stages in the formation of DNA-staining material are seen in figure 27. Aggregation of this amorphous material into larger clumps appears in figure 28.

Enzyme Digestion

Treatment of the cells, containing type-1 or type-2 inclusions, with DNase for 1 hour fails to remove the inclusions. The staining for DNA remains after staining with acridine orange, as shown in figure 29, on which two type-1 inclusions may be seen. Fluorescence of normal nuclear DNA is invariably prevented by the same treatment. Predigestion of the cells with pepsin for 1 hour, after treatment with DNase for 1 hour, removes the DNA-staining inclusions (fig. 30), while treatment with pepsin alone fails to remove the DNA staining, though it changes from yellow to copper, possibly due to acid hydrolysis of the DNA (*10*).

Histochemistry

The nuclear inclusions of both types stain by the Feulgen technique, first a delicate pink that becomes lavender-pink as the infection proceeds. When counterstained with light green, much Feulgen-negative material is seen in the nuclei of cells on the 3d to the 8th day after infection. This staining often persists even when an inclusion is present. The type-1 inclusions stain lavender-pink with Feulgen and are surrounded by a clear halo, which in turn is surrounded by Feulgen-negative material and Feulgen-positive granules (figs. 31 and 32). The type-2 inclusions also stain lavender-pink by the Feulgen method, as shown in figures 33 and 34, in which there are granules staining pink and Feulgen-negative material. A comparison between an infected and noninfected nucleus may be seen in figure 33.

Electron Microscopy

In the present study a number of adjacent thick and thin sections have been examined by means of the light and electron microscope. However, it was more convenient to study the infected cells by phase microscopy and the whole cell mounts by a variety of histochemical procedures and then fit the electron micrographs into the stages of the development of infection, to ascertain the submicroscopic structure of the various stages. Detailed results of this comparative study will be presented in a subsequent paper (*7*).

A comparison of the appearance of a type-1 inclusion, which stained lavender-pink by the Feulgen method, with the appearance of the inclusion in an adjacent thin section, as seen in the electron microscope, is shown in figures 35 and 35a. This inclusion (fig. 35a) contains virus particles arranged in a regular array (fig. 35). This arrangement of virus particles will be discussed in greater detail in (*7*). In addition, the characteristic virus particles are scattered between the considerably enlarged chromatin of the nucleus. There appears to be no doubt that the type-1 inclusions are of viral origin.

A comparison of the appearance of type-2 inclusion, as seen by phase microscopy, with that of a similar inclusion, as revealed in the electron microscope, is shown in figures 36 and 36a. Gray material scattered among the dense particles of chromatin (fig. 36a) is composed of the characteristic particles (fig. 36), which are shown at higher magnification in figure 37. The proximity of virus particles to the enlarged chromatin may be noted. The appearance of a similar inclusion may be seen in acridine-orange-stained preparation (fig. 26). Therefore, the inclusions described as type-2 are also of viral origin.

Bioassays

The results of bioassays have shown no tumor-inducing activity present in the polyoma-infected cell cultures before the appearance of the earliest

TABLE 1.—Tumor-inducing activity of tissue-culture fluid from polyoma-infected mouse-embryo cultures*

Dilution	Number hamsters alive at earliest tumor appearance (for each dilution)	Number hamsters with tumors	S.S.	Liver	Lung	Heart	Med.	H.D.	Earliest age of tumor appearance (days)	Average age of tumor appearance (days)
10^{-1}	11	9	8	2	2	1	1	2	44	55
10^{-2}	11	8	8		2			2	50	63
10^{-3}	15	3	3					1	77	84
10^{-4}	13	6	5	1				5	55	63

*S.S. = subcutaneous sarcoma.
Liver = hemangioma.
Lung = sarcoma.
Heart = sarcoma.
Med. = lymphosarcoma mediastinal.
H.D. = hemorrhagic disease.

visible change in the infected cells. A comparatively high-tumor-inducing activity was found in tissue-culture fluids, when the described changes were seen in the majority of cells (*see* table 1).

DISCUSSION

In this study, the cytological features observed in mouse-embryo cells, after infection with the polyoma virus, occurred with a high degree of reproducibility in several dozen experiments. No changes observed in the polyoma-infected cells were seen in cells of the control cultures. Although a number of strains of polyoma virus were used, the same characteristic changes were found in the infected cells. The time of appearance of the changes, however, varied somewhat in the infection with polyoma virus of different strains.

The interpretation of changes observed in the cells infected with a virus becomes more accurate if the changes seen with the phase microscope can be correlated with those after histochemical stains are used and with those observed in the electron microscope. One of the changes in viral infections is the formation of inclusions in the nucleus or cytoplasm. In this paper, inclusions have been observed in the nuclei and occasionally in the cytoplasm of mouse-embryo cells infected with polyoma virus.

Inclusions in the nuclei of cells infected with the polyoma virus could be characterized as viral inclusions only on the basis of observation of virus particles within these inclusions. It is possible with the help of the electron microscope to ascertain the true character of the inclusions that develop within the nucleus or cytoplasm of virus-infected cells. Information has been obtained on the fine structure and chemical composition of the inclusions in polyoma-infected mouse-embryo cells. This evidence is based on the correlation of the results obtained by phase and fluorescence microscopy, cytochemistry, and electron microscopy.

The morphological changes seen with the phase microscope in the nuclei of mouse-embryo cells in tissue cultures infected with the polyoma virus correspond to changes described as being associated with viral proliferation (*11*). Among these changes are the enlargement of cells, alterations in size and shape of the nucleolus, rearrangement of chromatin, a progressive increase in the size of intranuclear inclusions either in a diffuse, amorphous form, or in large, dense masses, and the appearance of inclusions in the infected cells. However, the presence of virus particles within an inclusion, either intranuclear or intracytoplasmic, appears to be the criterion for the determination of the origin of an inclusion that can then be described as an inclusion of viral origin.

In this study of mouse-embryo cell cultures infected with the polyoma virus, changes have been observed in the nuclei of cells, described as type-1 and type-2 inclusions. The type-1 inclusions were found much less frequently than type-2 inclusions. As seen in the phase microscope, they consist of homogeneous material, surrounding or displacing the

nucleolus, composed of one large or of several small, dense masses. By the Feulgen method, these inclusions stain at first delicate pink; as they enlarge and become more dense, as seen by the phase-contrast microscope, they stain lavender-pink with Feulgen (figs. 31 and 32). Similar changes have been described in the nuclei of cells of kidneys of animals infected with the polyoma virus (*12*). However, in the nuclei of other kidney cells acidophilic Feulgen-negative inclusions have also been found (*12*). They may represent a stage in the development of the Feulgen-positive inclusions observed in other nuclei. Enlargement of the nuclei of cells from kidneys of polyoma-infected mice with heavy strands and granules of chromatin scattered throughout the nucleoplasm has also been described (*12*). These changes appear to be similar to those observed in the polyoma-infected mouse-embryo cells, described as type-2 inclusions, found in a large number of cells and composed of dense strands or amorphous patches of gray material, filling the nucleoplasm. The type-2 inclusions, when stained by the Feulgen method, show similar staining properties to those observed in type-1 inclusions. At the height of the infection, the dense strands, or amorphous patches, become larger and darker, as observed by the phase microscope, and change from pink to lavender (fig. 34) or frankly Feulgen-negative (fig. 33). This change is of interest and may not be due to the green counterstain obscuring the Feulgen color, as Feulgen-positive granules are still present, surrounding the type-1 inclusions (figs. 31 and 32) or scattered within the amorphous patches (fig. 33) or the network (fig. 34) of type-2 inclusions. The significance of this observation cannot be ascertained at this time. It may indicate various stages of virus formation within the inclusions. Electron-microscope studies of the intranuclear changes involved in polyoma-virus formation, which will be described in (*7*), appear to support this possibility.

In the nuclei of cells with type-1 inclusions, the nuclear membrane appears thickened and sharply outlined (figs. 1 and 9), chromatin showing occasionally margination, which is much more pronounced in type-2 inclusions (figs. 14 and 15). In spite of intensive search, no transitional stages were observed between the two types of nuclear changes. While the nuclei of neighboring cells occasionally contained one of the two types of inclusions (fig. 19), binucleated cells as a rule showed only one, usually the type-2. Both types of nuclear changes appeared practically within the same interval of time after infection of mouse-embryo cells grown in tissue culture. However, at later intervals of time after infection, type-2 inclusions increased in number at a much faster rate than type-1 inclusions, the latter remaining consistently in minority. Daily observation by phase microscope showed variation in shape of type-1 inclusions, gradual inincrease in size, the inclusions almost filling the nucleus before cell death. Similar increase in size and in density was observed in the filamentous or amorphous material of type-2 inclusions before cell death.

The difference in appearance of intranuclear inclusions in polyoma-infected mouse-embryo cells raises the question: Are they the result of infection of different cell types or are they produced by the same virus?

It is difficult to answer the first part of the question in view of the mixed-cell population in cultures of mouse-embryo cells. Electron microscopy provides an answer to the second part. Virus particles of similar size and appearance have been found in both types of inclusions (figs. 35, 36, and 37). While morphological similarity need not indicate that the virus particles represent the same virus, the evidence is strongly suggestive from the results of cytochemical studies of these inclusions, which will be discussed later. Information about the type of cells showing the different inclusions may be obtained from clones of cells from mouse-embryo cultures infected with the polyoma virus and from biological tests of these cells showing one of the two changes. There is no doubt, however, that both types of inclusions are of viral origin as they show the presence of characteristic virus particles.

Enlargement of the nuclei showing the described changes was frequently observed, the nuclei occasionally increasing up to 10 times in size, compared with the nuclei of apparently unchanged cells. Enlargement of the nucleoli, often bizarre-shaped and vacuolated, was frequent, though it could not be correlated with the appearance of inclusions.

Cytochemical changes in polyoma-infected mouse-lymphoma cells grown *in vitro* were reported by Love and Rabson (*4*). They described enlargement of cells and the appearance of abnormal nuclear vacuoles which stained for ribonucleoprotein (RNP). On the basis of the changes observed in the cells stained with toluidine blue-molybdate (TBM), they concluded that normal nuclear mechanism of RNP synthesis was diverted into the process of polyoma-virus replication (*4*). No evidence of an increased synthesis of DNA was found in this study. Love and Rabson (*13*), in a recently published and particularly pertinent study on the cytochemistry of nucleic acids in murine lymphoma cells infected with polyoma virus *in vitro*, have observed essentially similar lesions to those described in the present study. By using TBM, they were able to distinguish nucleolar RNP from other intranuclear RNP. They found that the RNP of the *pars amorpha* of the nucleolus diffuses, fills vacuoles in the nucleus, and gives rise to dense masses of material. The enlargement of the nucleus and nucleolus, as the earliest observed change, was combined with the appearance of intranuclear Feulgen-positive homogeneous areas that were also stained by TBM. Since the intensity of staining of these areas by TBM was unaffected by ribonuclease (RNase) digestion, the authors concluded that they did not contain RNA at this stage. These areas seem to correspond to the type-1 inclusions described here. Later, small Feulgen-positive inclusions were noted; these appeared to incorporate RNP of nucleolar type. As the RNA in the inclusions increased, the Feulgen reaction decreased until eventually the inclusions were Feulgen-negative. They were, however, stained by TBM after digestion with RNase, due to the presence of DNA. Love and Rabson were not sure of the relationship between the homogeneous Feulgen-positive areas and the inclusions that appeared later. The present study suggests that both consist of viral particles, since a similar reduction in the intensity of

the Feulgen reaction was observed during the development of the type-1 inclusions. Finally, Love and Rabson noted that RNP of nucleolar type disappeared and a new type of RNP was demonstrated in the Feulgen-negative inclusions. The intensity of staining of the final inclusion by TBM was reduced by RNase, and all staining was abolished by digestion with RNase followed by DNase. The authors concluded that both DNP and RNP were present. The failure to stain by the Feulgen procedure was interpreted as caused by physical rather than chemical interference by viral protein with the staining reaction. The increase in susceptibility of the viral DNA to DNase after digestion with pepsin would suggest that viral DNA is less accessible to various experimental treatments than the DNA of chromatin by virtue of its protein coat. Inclusions were seen in the cytoplasm of cells showing advanced nuclear changes. Protein was present in association with nucleic acid in the homogeneous Feulgen-positive areas and in the inclusions at all stages.

In the present study, DNA was detected in the early stages of inclusion formation, both in the cytoplasm and the nucleus, as revealed by Feulgen-positive staining. This gradually changed into lavender or lavender-pink, or frankly Feulgen-negative, in the final stages of infection. This change was not due to counterstaining with light green, as Feulgen-positive granules could easily be detected at this stage. This is especially clear in figure 33, in which the Feulgen-positive granules are chromatin granules and the Feulgen-negative material corresponds in location with virus particles, as will be discussed in detail in (*7*). There is no doubt that the inclusions change color as the infection proceeds, as observed by Rabson and Love (*13*). This change in Feulgen-staining of the inclusions will have to be further investigated before an explanation can be provided. Variations with the Feulgen-staining may be attributed to the fact that the inclusions undergo a series of progressive changes entailing different functional characteristics with the development, multiplication, and dispersal of the virus.

In the present study, DNA was demonstrated in both types of intranuclear inclusions by means of fluorescent microscopy with the use of acridine orange dye (*9*). No RNA was detected in the infected cell nuclei, except for the pink or orange-pink staining of the nucleoli with acridine orange. This does not necessarily indicate absence of RNA in the nucleus. If the infection with polyoma virus leads to the formation of RNA, the fluorescence technique used in this study may not be sufficiently sensitive to identify it. Further studies are planned to investigate the part played by RNA in the polyoma-cell relationship.

The staining for DNA with acridine orange, in itself, does not indicate whether the nucleic acid is of host or viral origin. The specificity of staining may, however, be assessed by pretreatment with enzymes, such as pepsin and nucleases (*10*, *14–16*). While treatment with DNase removes the yellow staining for DNA from changes due to the nonspecific reaction of the host cell, it fails to remove the staining from changes of viral origin. Pretreatment with pepsin, followed by digestion with DNase,

removes the DNA-staining of viral inclusions. In the present study, treatment of the DNA-staining nuclear inclusions with DNase failed to change the yellow or yellowish-green fluorescence of type-1 and type-2 intranuclear inclusions in polyoma-infected mouse-embryo cells. Treatment with DNase also failed to remove RNA, as evidenced by the brilliant red of the cytoplasm. No RNase could be detected in the DNase preparation in tests, based on the slightly modified acid-soluble method of Laskowski (*17*) and on the McDonald modification of the Kunitz method (*18*). Digestion with pepsin (*p*H 2.0) also failed to remove the yellow-staining inclusions in the nucleus and the cytoplasm, and the cytoplasm and nucleolus stained red or orange-red, as before treatment with pepsin. Pretreatment with pepsin, followed by DNase digestion, removed the fluorescent DNA material from the inclusion bodies. Pretreatment of the inclusions with pepsin appears to break down the protein coat, exposing the viral DNA to treatment with DNase. The results of these studies indicate the viral nature of the inclusions observed in polyoma-infected mouse-embryo cells. These results have been substantiated by the electron-microscope observations of characteristic virus particles, within both types of inclusions, described in the present study (figs. 35 through 37). The size of the virus particles will be discussed in (*7*). Similar particles have previously been reported in polyoma-infected mouse-embryo cells (*19–22*). The results of treatment with pepsin and DNase of preparations stained with acridine orange indicate that the particles seen in the inclusions present in polyoma-infected mouse-embryo cells are those of a DNA-containing virus.

During the final stages of infection with the polyoma virus, inclusions have been observed in the cytoplasm of the infected mouse-embryo cells (figs. 23 and 24). These inclusions of varying size and shape stain yellow for DNA with acridine orange, but some stain orange and some greenish-yellow. The staining is removed by digestion with pepsin and DNase, but not by DNase or pepsin alone. This indicates the viral nature of the inclusions, and their fine structure will be described in (7).

Investigations of polyoma-infected cells by the fluorescent antibody technique have revealed a striking similarity between the appearance of the specific antigen and the site of infection (*5*, *6*). These studies support the observations on the viral nature of the changes in the nucleus and cytoplasm of the infected cells.

Allison and Armstrong (*23*) have recently reported their findings in polyoma-infected mouse-embryo and "L" cells grown *in vitro* and stained with acridine orange. They described the appearance of abnormal DNA foci in the nuclei, which corresponded to an increase in the titer of the polyoma virus in the infected cultures. They concluded that the changes are caused by polyoma virus and that it contains DNA. These observations are similar in the main aspects to those reported in the present study.

Evidence has recently been presented which indicates that polyoma virus is DNA-carrying (*24*, *25*), though there are data available indicating that RNase interferes, under certain conditions, with the activity of

nucleic-acid extracts of polyoma-infected mouse-embryo tissue cultures (*26*). This, in itself, need not contradict the findings on polyoma virus as a DNA virus, in view of the observations on vaccinia virus reported by Tamm and Bablanian (*27*). From the recent studies on the relationship between changes in DNA and RNA or RNP metabolism in polyoma-virus infection (*13*), it appears that nucleolar RNA participates in the formation of polyoma-induced inclusions.

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PLATE 14

Phase photomicrographs

FIGURE 1.—Appearance of C3H/An/Dm mouse-embryo cell, grown in tissue culture, 2 days after infection with SE polyoma virus. Enlarged nucleoli, surrounded by clear zone, is seen. × 810

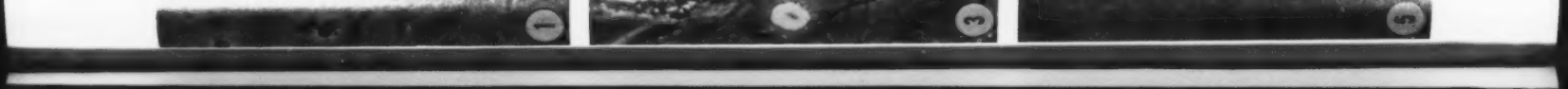
FIGURE 2.—Appearance of C3H/An/Dm mouse-embryo cell, 3 days after infection, which shows bizarre-shaped nucleoli and small, dark granules in the nucleus; cytoplasm showing granulation and vacuolization. × 630

FIGURE 3.—C3H/An/Dm mouse-embryo cell, 3 days after infection, which shows bizarre-shaped nucleoli and 2 inclusions in nucleus. × 570

FIGURE 4.—Similar cell, showing inclusion within nucleus and 2 nucleoli. × 630

FIGURE 5.—C3H/An/Dm mouse-embryo cell, 3 days after infection, which shows vacuoles in the cytoplasm filled with material of lesser density than surrounding cytoplasm. In some vacuoles dark granules are seen. × 630

FIGURE 6.—Similar cell, 3 days after infection, which shows increased granulation of cytoplasm. × 810



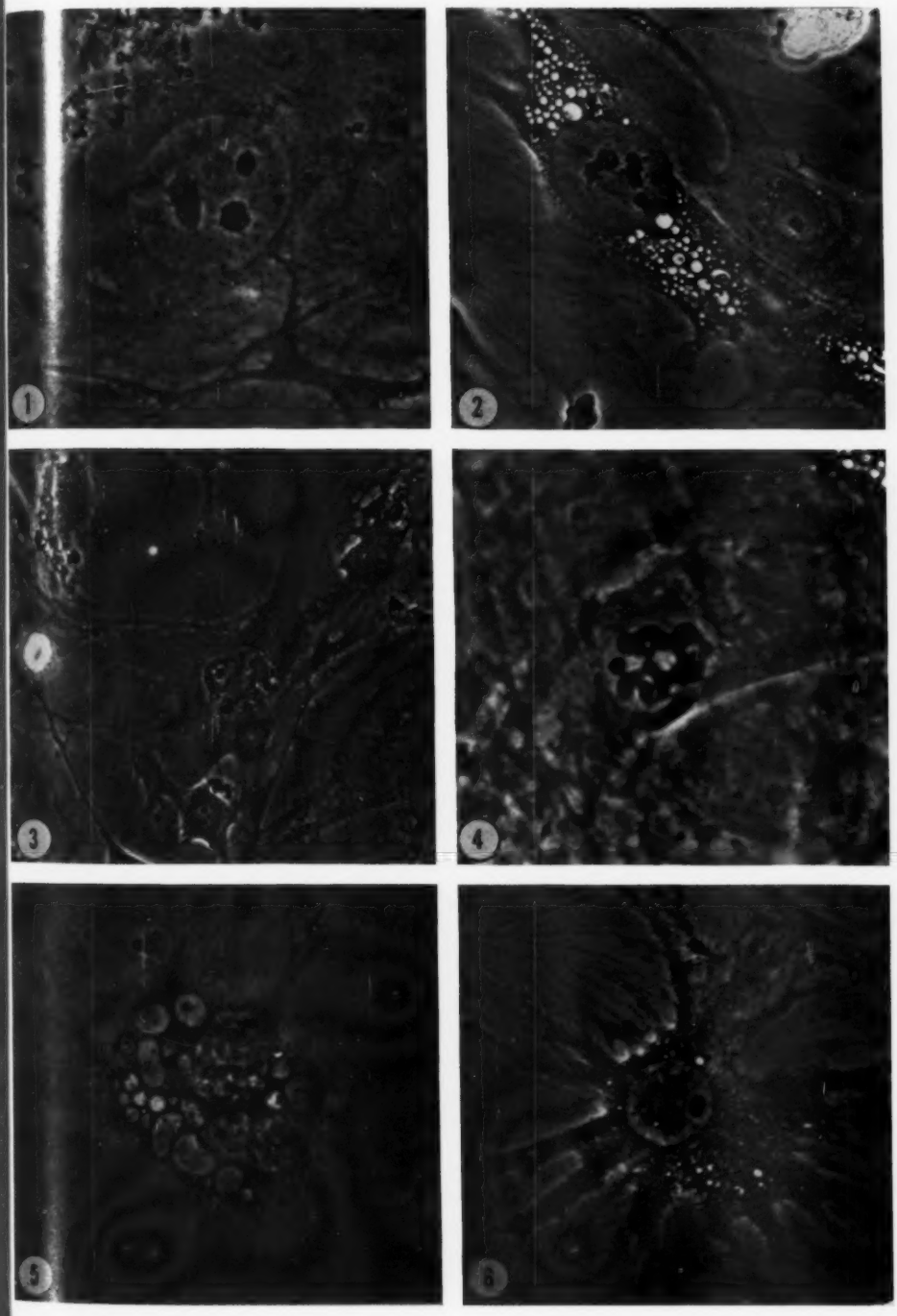


PLATE 15

Type-1 nuclear inclusions—phase photomicrographs

FIGURE 7.—Appearance of nuclear inclusion in a C3H/An/Dm mouse-embryo cell 4 days after infection. Three nucleoli are seen. × 630

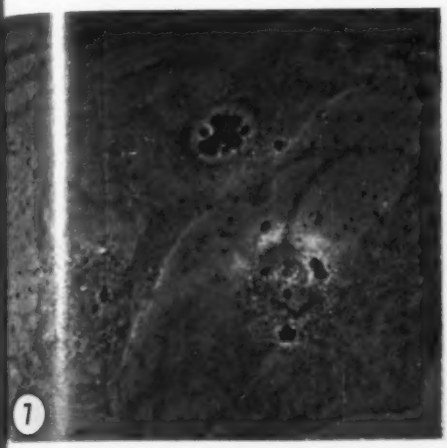
FIGURE 8.—Inclusion surrounding nucleolus in a C3H/An/Dm mouse-embryo cell 5 days after infection. Increased granulation and vacuolization of cytoplasm. × 630

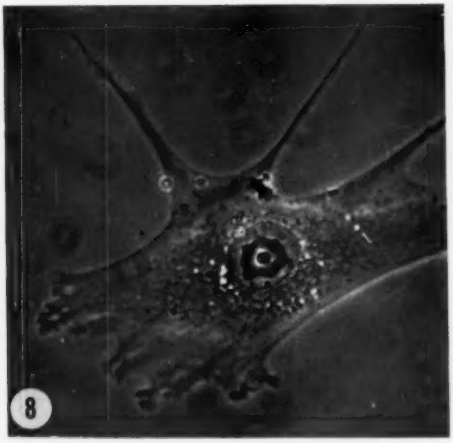
FIGURE 9.—Cell showing dark nuclear inclusions, which is surrounded by a clear zone, 5 days after infection with SE polyoma. Nuclear membrane is distinctly outlined. × 1,800

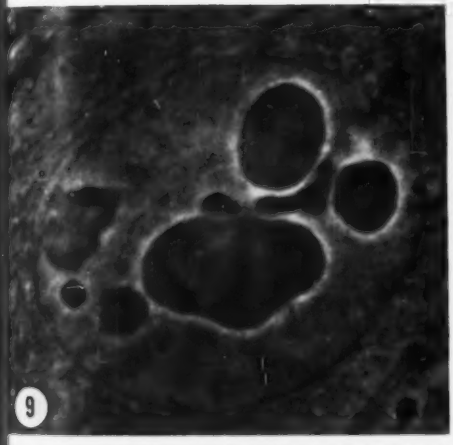
FIGURE 10.—Cell showing 2 inclusions in nucleus and chromatin aggregation along nuclear membrane 5 days after infection. × 1,800

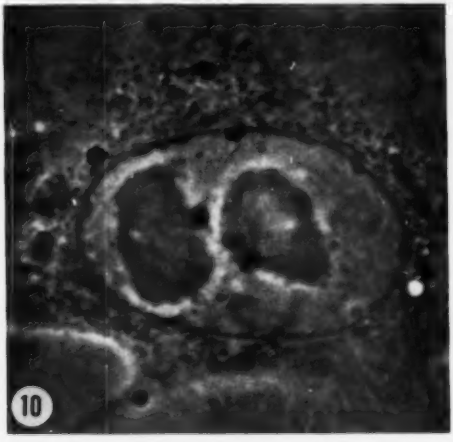
FIGURE 11.—Appearance of similar type of nuclear inclusion in a cell, 5 days after infection. × 900

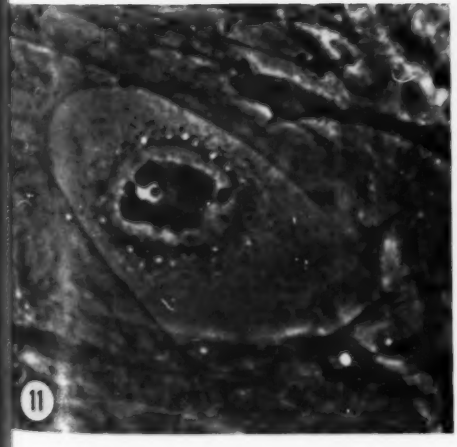
FIGURE 12.—Inclusion, surrounded by halo, which almost fills nucleus of a cell, 5 days after infection. Uninfected cells are seen in the vicinity. × 810











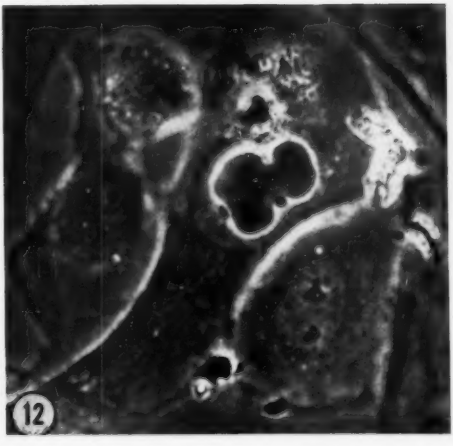


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PLATE 16

Type-2 nuclear inclusions—phase photomicrographs

FIGURE 13.—C3H/An/Dm mouse-embryo cell, 5 days after infection with SE polyoma virus, which shows nucleus with strands and patches of gray material. × 630

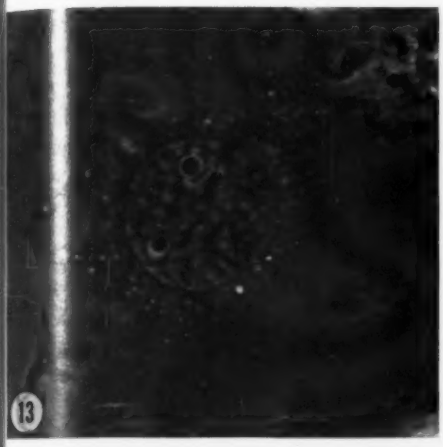
FIGURE 14.—Similar cell, 4 days after infection, which shows aggregation of chromatin around nuclear membrane and strands of gray material in association with chromatin and nucleoli. × 810

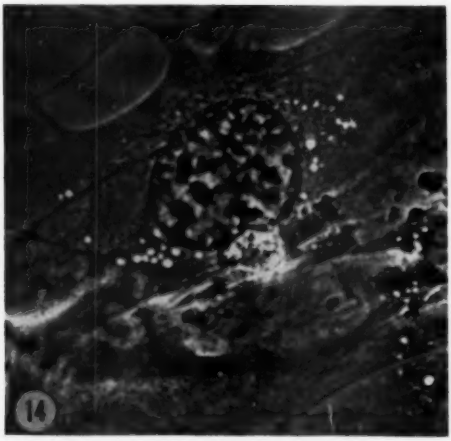
FIGURE 15.—Similar cell, 6 days after infection, which shows increased density of gray material filling most of the nucleus. Nucleolus surrounded by halo and aggregation of chromatin around nuclear membrane. × 1,580

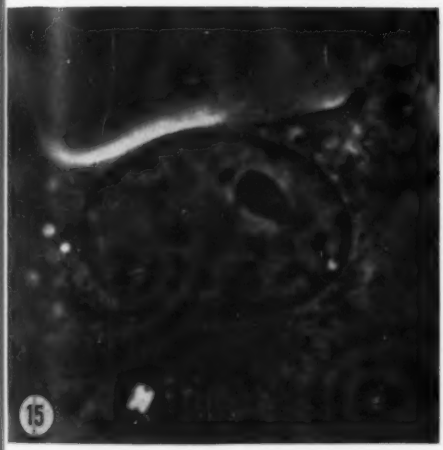
FIGURE 16.—Cell, 7 days after infection, which shows scattered amorphous gray material in nucleus, vacuolated nucleolus, and increased granulation of cytoplasm. × 630

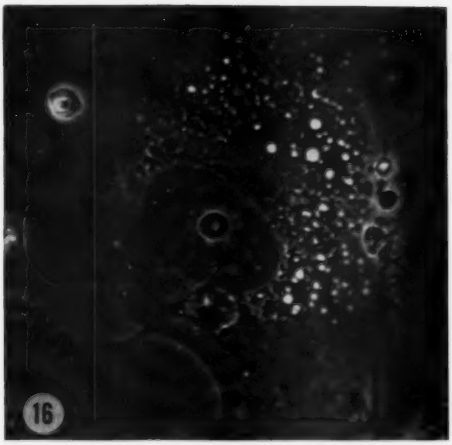
FIGURE 17.—Mouse-embryo cell, 7 days after infection, which shows increased density of enlarged dense amorphous material, with lighter gray material within network. Cytoplasm is filled with small, dark granules and vacuoles. × 1,580

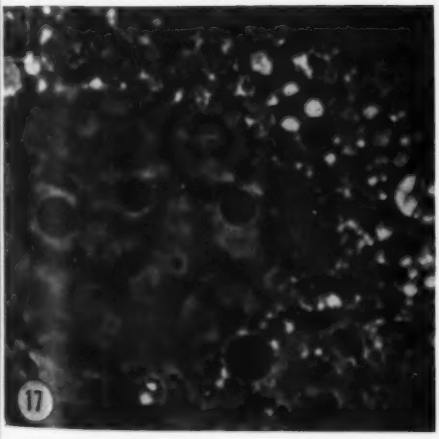
FIGURE 18.—Mouse-embryo cell, 9 days after infection, which shows nucleus entirely filled with dark amorphous material. Similar nucleus is present in *upper right* corner. × 1,580











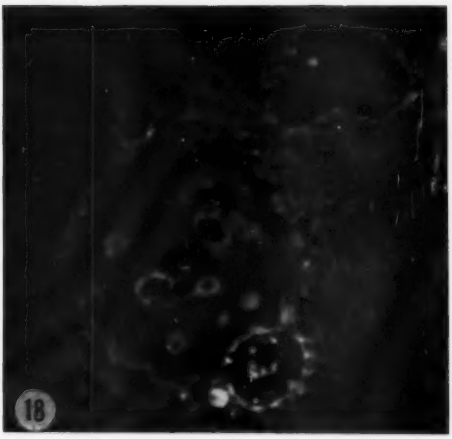


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PLATE 17

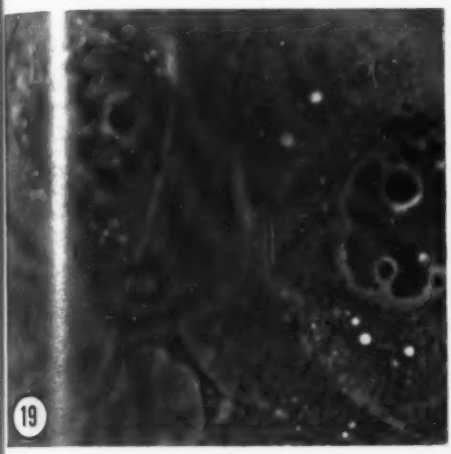
Phase photomicrographs

FIGURE 19.—Nuclei of 2 mouse-embryo cells, one on *right* showing type-1 and one on *left*, type-2 inclusions, 7 days after infection with SE polyoma virus. × 1,580

FIGURE 20.—Characteristic grapevine appearance of mouse-embryo cell, 14 days after infection with SE polyoma virus. × 630

FIGURE 21.—Control mouse-embryo culture. Appearance after 7 days. × 720

FIGURE 22.—Control mouse-embryo culture. Appearance after 9 days × 720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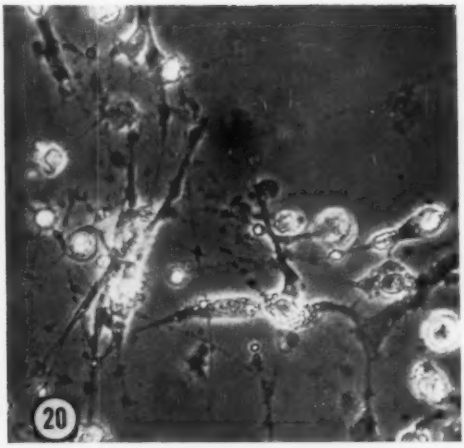
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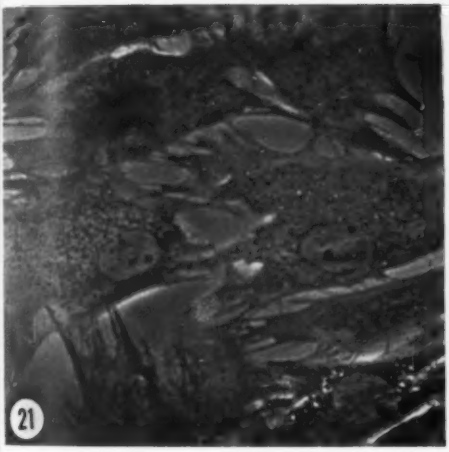
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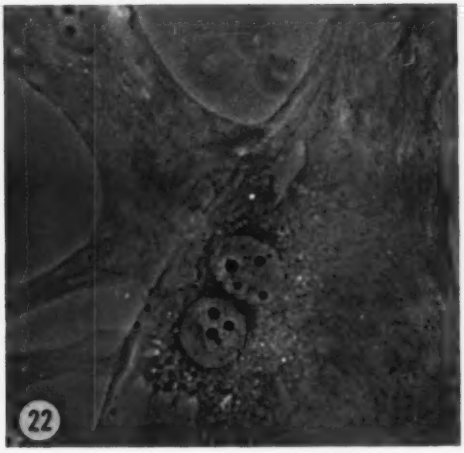
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PLATE 18

Appearance of SE polyoma-infected mouse-embryo cells, after staining with acridine orange fluorescent dye—black-and-white reproductions of fluorescence photomicrographs

FIGURE 23.—Appearance of 2 cells, 5 days after infection with virus. In *top right* corner nucleus showing type-1 inclusion staining yellow for DNA, with similarly stained material around nuclear membrane. Cytoplasm of this cell shows 3 inclusions staining greenish-yellow and yellow. Cell with smaller nucleus shows type-2 intranuclear inclusion and several inclusions in cytoplasm. All inclusions stain yellow. × 500

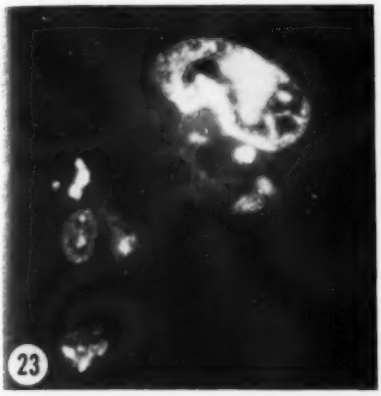
FIGURE 24.—Nucleus, marked by *arrow*, shows large, dense, yellow-staining type-1 inclusion. The cytoplasm of one of cells contains 2 smaller, yellow-staining inclusions, marked by *arrows*. Five days after infection. × 380

FIGURE 25.—Amorphous yellow-green DNA-staining material present between 2 nucleoli staining orange-red. A number of small yellow-staining granules also present in nucleus, 5 days after infection. × 1,620

FIGURE 26.—Cell in *center* shows nucleus with type-2 inclusion. Chromatin around nuclear membrane and amorphous material in nucleus stain yellow for DNA. Apparently uninfected nuclei are in surrounding area. Five days after infection. × 740

FIGURE 27.—Three nuclei, each displaying what may be described as progressive steps in development of type-2 inclusion. Nucleus on *left* with amorphous material staining light yellowish-green; nucleus on *right* with material stained deeper yellowish-green and red-staining nucleolus; nucleus in *center* is intensely stained for DNA. Culture 6 to 9 days after infection. × 430

FIGURE 28.—Nucleus in *center* shows dense yellow staining in last stages of type-2 inclusion formation. Culture 6 to 9 days after infection. × 650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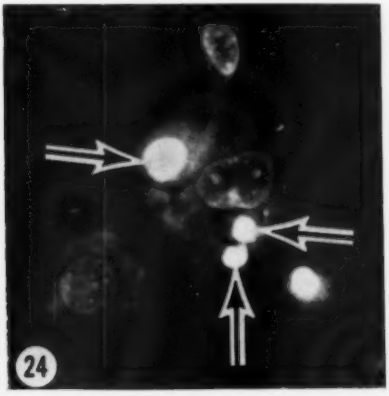
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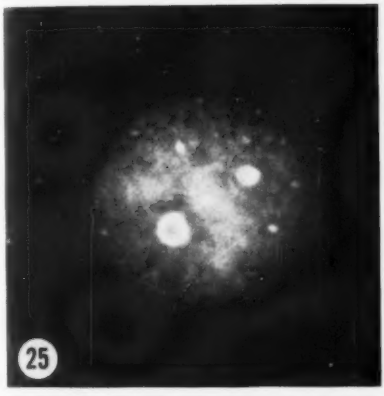
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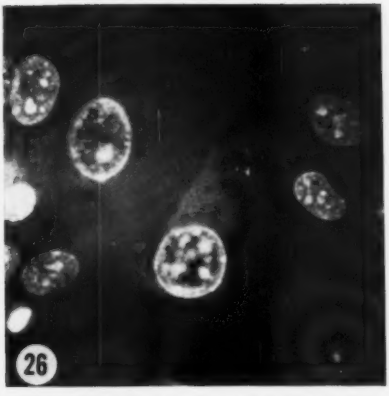
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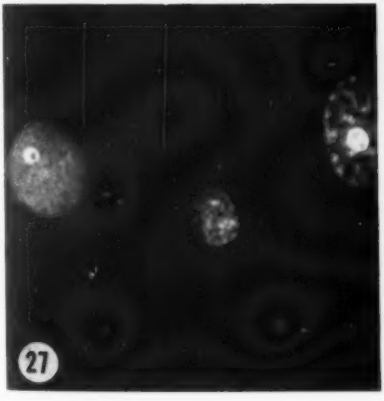
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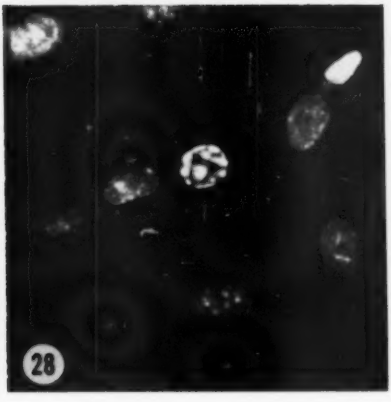
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PLATE 19

Figures 29 and 30 are black-and-white reproductions of fluorescence photomicrographs. Figures 31 through 34 are black-and-white reproductions of colored photomicrographs.

FIGURE 29.—Appearance of C3H/An/Dm mouse-embryo cells, infected with SE polyoma, showing 2 type-1 inclusions, after staining with acridine orange and pretreatment with DNase. The inclusions stain yellow for DNA. × 430

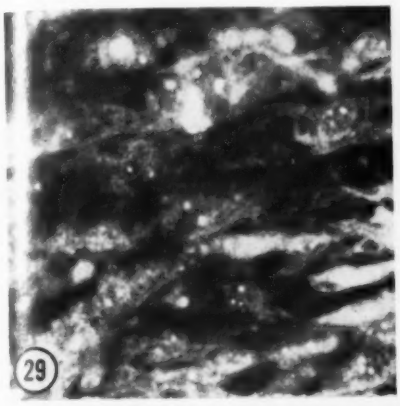
FIGURE 30.—Appearance of polyoma-infected mouse-embryo cells stained with acridine orange after treatment with pepsin and DNase. The yellow-staining inclusion in nucleus in *center* of field has been removed. × 410

FIGURE 31.—Appearance of nucleus with type-1 inclusion, after staining by Feulgen method. The lavender-pink inclusion may be seen surrounded by clear zone. Fuelgen-positive granules (chromatin) also present in nucleus. × 1,300

FIGURE 32.—Another example of type-1 intranuclear inclusion within enlarged nucleus, staining lavender by Feulgen procedure. Feulgen-positive granules are present in nucleus. Uninfected nuclei in the vicinity. Feulgen stain. × 740

FIGURE 33.—Nucleus on the *right* shows type-2 inclusion, composed of amorphous strands stained lavender-pink by Feulgen method, with Feulgen-negative patches of amorphous material and Feulgen-positive granules (chromatin). On *upper right* an apparently uninfected nucleus. × 1,300

FIGURE 34.—Another example of type-2 inclusion within nucleus, containing amorphous lavender-pink staining material. Numerous pink granules (chromatin) are scattered throughout nucleus. Feulgen stain. × 1,200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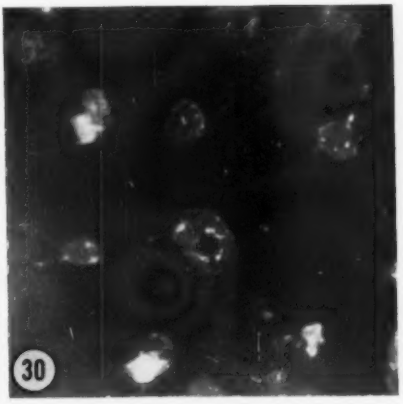
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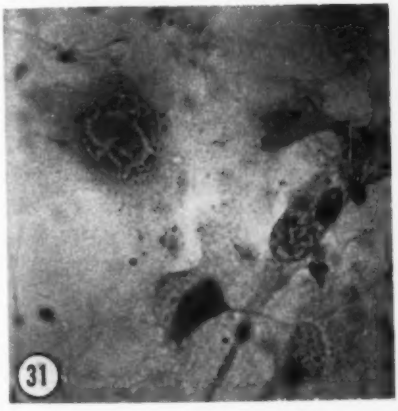
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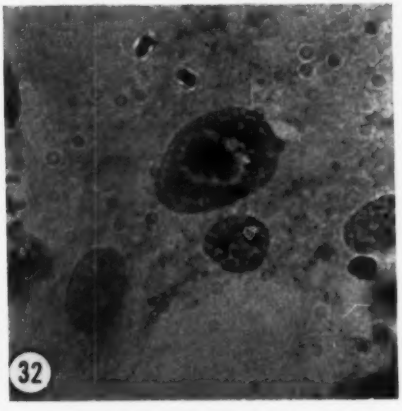
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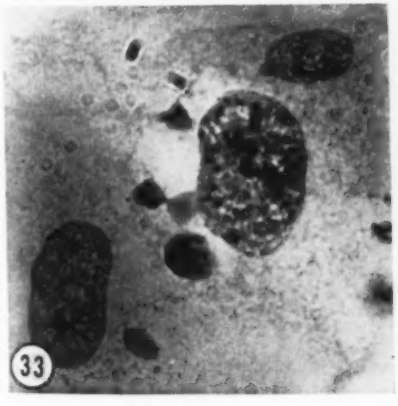
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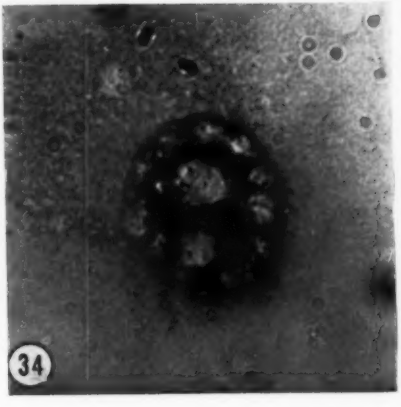
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PLATE 20

FIGURE 35.—Nucleus of SE polyoma-infected C3H/An/Dm mouse-embryo cell showing considerable increase in chromatin with type-I inclusion with virus particles in orderly array. Numerous virus particles are scattered between chromatin in *upper* part of nucleus. Electron micrograph. × 35,000

FIGURE 35a.—Appearance of nucleus in adjacent section, after staining with Feulgen. The lavender-pink inclusion appears darkly stained. Photomicrograph. × 2,000

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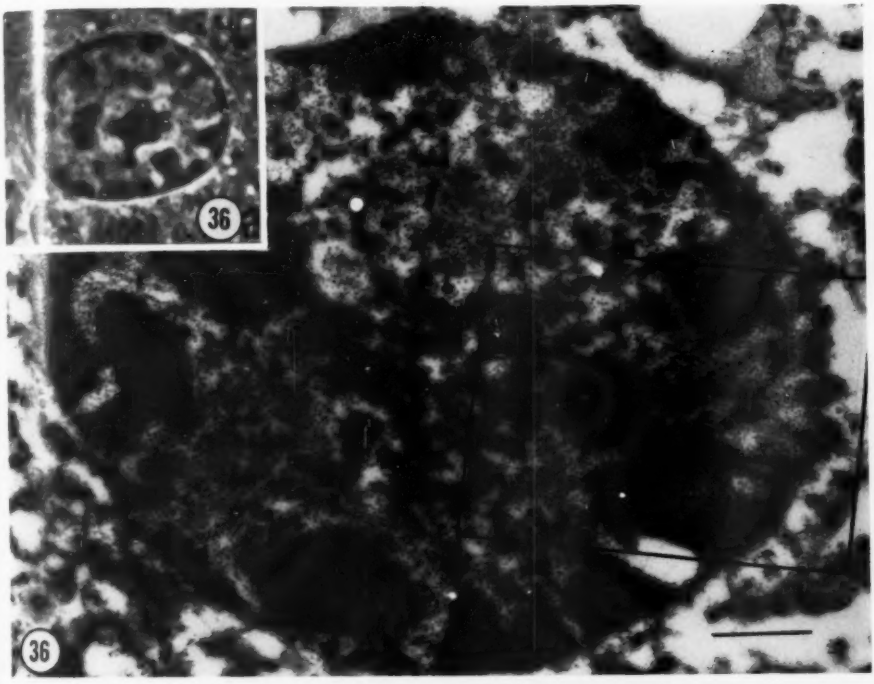
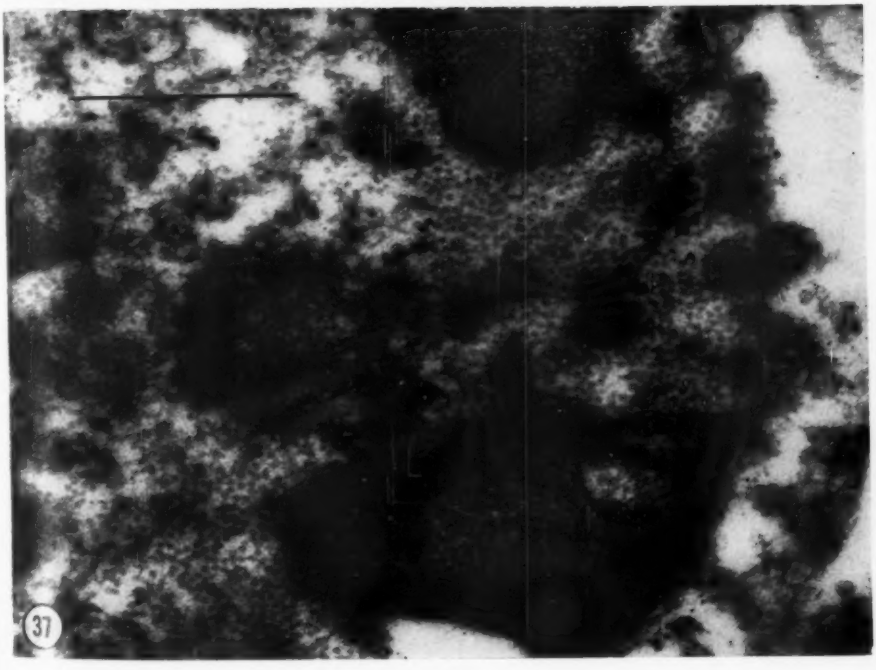
PLATE 21

FIGURE 36.—General appearance of enlarged nucleus of SE polyoma-infected C3H/An/Dm mouse-embryo cell, showing type-2 inclusion. Numerous virus particles present among strands of enlarged chromatin. Nucleolus may be seen in *center*. Electron micrograph. × 16,000

FIGURE 36a.—Appearance of similar nucleus containing type-2 inclusion. Phase photomicrograph. × 1,100

FIGURE 37.—Part of figure 36, marked by rectangle, showing virus particles scattered among chromatin. Electron micrograph. × 37,000





593953—61——16

Liver Cell Proliferation in X-Irradiated Rats After Single and Repetitive Partial Hepatectomy [1]

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SUMMARY

The behavior of hepatic tissue of previously irradiated rats, after repeated stimuli to cell proliferation, has been studied. Less than 10 percent of the mitoses in regenerating liver of nonirradiated rats was visibly aberrant, whereas 75 to 80 percent of the mitoses in postirradiation animals was abnormal. These abnormal mitoses persisted in the same frequency after repeated partial hepatectomies and for as long as 17 weeks after the initial exposure to X ray, in spite of the fact that the partial hepatectomies resulted in extensive hepatic-cell replacement. Regeneration of livers of the irradiated animals was characterized by (*a*) tissue-mass restoration equal to the controls, (*b*) significant decrease in the peak mitotic index (50 to 60% below values in nonirradiated controls), and (*c*) a significant decrease in parenchymal cell numbers per microscopic field, *i.e.*, larger parenchymal cells.—J. Nat. Cancer Inst. 27:131–143, 1961.

PREVIOUS INVESTIGATIONS (*1*, *2*) of the effect of X rays on the mitotic process in mouse liver cells clearly demonstrate the presence of increased numbers of abnormal mitotic figures during CCl_4-induced liver regeneration. This increase in mitotic abnormalities was found after irradiation of resting, as well as of mitotically active, cells. Although quantitative studies of these aberrant mitotic figures were conducted under various conditions, there is little information concerning the persistence of X-ray-induced abnormalities through several repeated cycles of mitotic activity in livers subjected to several successive regeneration episodes.

The present investigation is primarily concerned with the effect of X irradiation on the nuclear mitotic apparatus in normal resting liver cells of mature rats. Since the mitotic activity of normal adult liver is almost nil, one may be fairly certain that the particular cells subjected to the X-ray beam are still present as such in the tissue, even a long time

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[3] The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

593953—61——17

after irradiation. By partial hepatectomy, it is possible to elicit an intense wave of mitoses, finally resulting in complete or almost complete recovery of the resected tissue mass within 10 to 14 days after surgery (*3*, *4*). Repetitive partial hepatectomy, then, should allow a quantitative analysis of the ability of "noninjured" cells (mitoses not visibly aberrant) to replace liver mass by proliferation, as well as the ability of the damaged cells (showing visibly aberrant mitoses) to survive through successive regeneration cycles. The present report concerns observations on the previously irradiated rat liver after single and repetitive stimuli (partial hepatectomy) to cell proliferation, and will present data on the regenerative behavior of such a tissue with respect to restored liver weight, mitotic index, and the incidence of abnormal mitoses.

METHODS

Animals.—Male Sprague-Dawley rats (NRDL strain), 3 months old at the time of the first exposure to X rays and weighing 300 to 350 g, were used. They were maintained in air-conditioned quarters (74° F). Purina laboratory chow and water were available at all times.

Irradiation procedure.—One half of the animals for each experimental group was exposed to 2 whole-body doses of 400 r X ray (250 kvp, 15 ma; filter 0.5 mm Cu and 1 mm Al; HVL 1.4 mm Cu; TSD 40 in.; dose rate 29.8 r/min in air) spaced 3 weeks apart.

The rats being irradiated were placed in a special circular cage of Lucite, holding 20 rats in individual compartments. The cage was rotated in a horizontal plane (3 rpm) under the X-ray tube during the radiation exposure. The control animals were subjected to sham irradiation in the same setup. The number of animals per group is designated in table 1.

Surgical procedure.—To minimize possible variations due to the diurnal cycle of the rat (*5*), surgery was scheduled so that all animals would be killed in the morning hours. The procedure of (*6*) was used for the partial hepatectomies. In the animals subjected to a single partial hepatectomy, approximately 60 to 70 percent of the total liver mass was removed. In those animals subjected to 3 successive partial resections, approximately 40 percent of the total liver mass was removed at each excision. The left lateral lobe, median lobe, and the 2 right lateral lobes were removed successively. Three weeks were allowed between each pair of partial resections to permit almost complete restoration of the original liver weight; at this time the mitotic activity of the tissue had returned to its value before surgery (*3*, *4*).

Histological techniques.—The animals were invariably killed during the morning hours. We exsanguinated the rats, under ether anesthesia, by severing the vena cava and vena porta, and two specimens were taken from each lobe of every liver—one cut parallel to, and the other at right angles to, the long axis of each lobe. Peripheral and central portions of the lobes were included in all tissue samples. The tissues were fixed in

Tellyesniczky's solution (ethyl alcohol, glacial acetic acid, and formalin) for 8 to 12 hours, dehydrated, embedded in paraffin, sectioned at 5 μ, and stained in Harris' hematoxylin and eosin.

Method of analysis.—Mitotic abnormalities and mitotic indexes were determined and counted under oil immersion ($\times$ 970). Although abnormalities (*see* description under "Results" for definition) were observed in all stages of mitoses, only those occurring during anaphase and telophase were quantitated, since abnormalities in these stages could be diagnosed with the greatest assurance. A minimum of 200 mitotic figures in anaphase and telophase for each of two lobes from every liver was evaluated for abnormalities. Results of such counts are expressed as the ratio of abnormal figures to the total number of figures viewed.

Mitotic index is expressed as the number of mitoses per 1,000 parenchymal cells counted. One hundred consecutive fields were examined on each slide, and all mitoses from late prophase to late telophase were counted in every field. On at least 1 section from each liver, all parenchymal cells in the 100 fields viewed were counted. In subsequent slides from the same liver, all mitoses in 100 fields were counted, but only the parenchymal cells in every fifth field were enumerated. Again, sections from at least 2 lobes of each liver were examined, and, in some cases, 100 fields were counted in several areas of the same lobe to assess regional differences in the distribution of dividing nuclei. With these procedures, a minimum of 3,000 to 4,000 cells/slide was viewed.

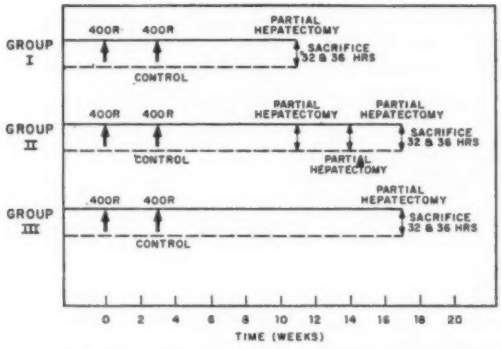
To assure a maximum number of mitoses in anaphase and telophase stages, we killed the animals 32 and 36 hours after the last partial hepatectomy. Since the mitotic peak is reached between 28 and 32 hours after subtotal hepatectomy (*3*), it was assumed that 32 hours would still be within the peak period for the control rats and that those animals killed 4 hours later would permit the detection of any delay in the mitotic peak of the irradiated rats.

Experimental design.—The design of the over-all experiment is shown in text-figure 1. It includes irradiated and sham-irradiated groups, subdivided further into 3 groups sustaining, respectively, a single partial hepatectomy 8 weeks after the end of irradiation, 3 successive partial hepatectomies as described, or a single partial hepatectomy at a time corresponding to the last of the 3 repetitive resections. Further subdivision into times of killing relative to the last resection follows from the procedures described.

Finally, to evaluate the ability of the irradiated animals to restore liver mass, a number of animals was maintained for 3 weeks after the third and final partial hepatectomy.

Statistics.—Standard deviations of all experimental points were calculated by estimation from the range of the individual values; P values for differences of means were calculated by the t test.

Relative cell size.—To estimate changes in relative size of liver cells in comparable specimens from livers subjected to the several sequences of experimental procedure, numbers of parenchymal nuclei for 100 random



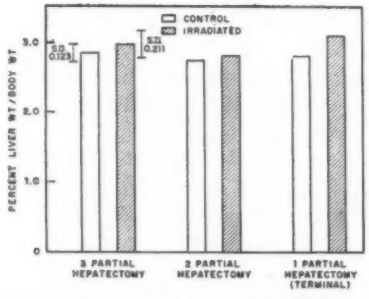
TEXT-FIGURE 1.—Experimental design of irradiation and partial hepatectomy schedule.

fields (oil immersion as before) were determined on histological sections prepared under standard conditions. The estimate of relative cell size can be made on either of two extreme hypotheses: If one assumes that the number of cell nuclei counted represents the total number of cells in a reproducible volume, the cell counts obtained are inversely proportional to the mean cell volume. If, on the other hand, one assumes that the sample represents the cells intercepting a specific plane surface laid through the tissue, then the cell counts are inversely proportional to the mean cross-sectional area, and by inference must be raised to the 1.5 power to convert the ratios obtained to cell-volume ratios. The "true" relation must lie between these extremes, probably more nearly approximating the former under the focusing procedure used.

RESULTS

Liver Weight/Body Weight

Text-figure 2 illustrates the liver-weight/body-weight ratio 3 weeks after the final resection for animals subjected, respectively, to 2 and 3 successive partial hepatectomies, or to one terminal partial hepatectomy at the end of the experimental period.



TEXT-FIGURE 2.—Liver weight/body weight 3 weeks after partial hepatectomy.

The liver-weight/body-weight ratio of the X-irradiated and of nonirradiated animals which had undergone 3 successive resections are 3.0 ± 0.2 and 2.9 ± 0.1 percent, respectively; the difference is not significant. Similarly, significant differences could not be shown for X-irradiated and control animals subjected to 2 successive partial hepatectomies, or to a single terminal partial hepatectomy. Standard deviations for all groups ranged between 0.15 to 0.20 percent.

Mitotic Index

The mitotic index for the livers of nonirradiated animals subjected to a single, initial partial hepatectomy (killed 32 hours after surgery) was 61 ± 17 as compared to only 28 ± 11 mitoses/1,000 parenchymal cells for the X-irradiated animals, a decrease of approximately 50 percent. Animals killed 36 hours after surgery showed a similar decrease in mitotic index after exposure to X irradiation, from 35 ± 13 for the controls to 20 ± 6.2 for the irradiated animals.

Histological samples of the livers of control and irradiated animals were taken at the time of the second and third partial hepatectomies to determine whether the mitotic index had been restored to normal values. The mitotic index for these animals was equal to that of nonhepatectomized controls (<1 mitosis/1,000 parenchymal cells) which indicated that proliferative activity of hepatic tissue had returned to preresection values within 3 weeks after subtotal hepatectomy.

The mitotic indexes for control and irradiated animals subjected to 3 successive partial hepatectomies were lower than for the animals subjected to a single partial hepatectomy. For the animals killed early, *i.e.*, 32 hours after surgery, the mitotic index of the nonirradiated controls was 38 ± 14, compared to 19 ± 12 for the irradiated groups ($P<0.01$). No significant difference could be detected between irradiated and nonirradiated animals killed at 36 hours (table 1).

To test the possibility that the animals may have recovered from the nuclear damage during a 17-week experimental period, a single partial hepatectomy was carried out 17 weeks after the start of irradiation on a group of animals (X-irradiated and nonirradiated) corresponding to group II. The mitotic indexes for the X-irradiated animals in this experiment, those killed 32 and 36 hours after surgery, were decreased by 40 percent below the mean for the control animals (table 1), and thus failed to show significant recovery.

Abnormal Mitoses

Mitotic abnormalities were observed in the metaphase, anaphase, and telophase stages of cell division. The abnormal metaphases could be primarily described as fragmentations of chromosomes, lagging chromosomes remote from the spindle, and malformed spindles. The primary distinguishing features of abnormal mitoses in the anaphase stages were chromosome bridges varying from a single broad band to multiple chro-

TABLE 1.—Mitoses/1,000 parenchymal cells 32 and 36 hours after partial hepatectomy*

	Nonirradiated		X-irradiated		*P*	
	32 Hours	36 Hours	32 Hours	36 Hours	32 Hours	36 Hours
Initial single partial hepatectomy	61.7 ± 17.9 (7)†	35.6 ± 13.8 (6)	28.9 ± 11.6 (8)	20.9 ± 6.2 (7)	<0.01	0.015
3 Partial hepatectomies	38.1 ± 14.9 (7)	37.9 ± 9.3 (7)	19.9 ± 12.9 (6)	31.9 ± 8.2 (5)	<0.01	0.15
Terminal single partial hepatectomy	62.9 ± 11.9 (7)	49.5 ± 13.3 (8)	37.8 ± 8.8 (7)	35.3 ± 6.3 (8)	<0.01	0.015

*All values reported are mean ± standard deviation.
†Numbers in parentheses = number of animals in each group.

TABLE 2.—Frequency of abnormal mitoses, as percent of all anaphase and telophase figures observed to be abnormal*

	Nonirradiated		X-irradiated		*P*	
	32 Hours	36 Hours	32 Hours	36 Hours	32 Hours	36 Hours
Initial single partial hepatectomy	4.1 ± 0.7 (7)†	8.8 ± 5.0 (6)	76.9 ± 15.0 (8)	82.1 ± 3.9 (7)	<0.01	<0.01
3 Partial hepatectomies	7.6 ± 1.4 (7)	7.4 ± 2.7 (7)	75.7 ± 9.3 (6)	81.2 ± 6.5 (5)	<0.01	<0.01
Terminal single partial hepatectomy	7.8 ± 3.1 (7)	4.6 ± 2.9 (8)	81.0 ± 10.4 (7)	78.7 ± 7.3 (8)	<0.01	<0.01

*All values reported are mean ± standard deviation.
†Numbers in parentheses = number of animals in each group.

matin bands between daughter nuclei. Often chromosome fragments or particles were observed scattered throughout the cytoplasm. The usual polar formation of two daughter nuclei was characterized at times by tripolar structure and at other times by total lack of discernible polarity.

In the telophase stage, the usual mitotic abnormalities consisted of an asymmetric distribution of chromatin material to the daughter nuclei, incomplete separation of the daughter nuclei usually connected by a single or several strands of chromatin material, and the formation of micronuclei in addition to the frequent appearance of chromosome fragments.

Less than 10 percent of the mitoses in regenerating liver of nonirradiated rats was visibly aberrant, regardless of whether they were subjected to a single or repeated partial hepatectomy. By contrast, however, 75 to 80 percent (mean 77.6%) of the mitoses in all postirradiation animals was classified as visibly abnormal. This was found to be similarly true for the groups of animals subjected to a single initial partial hepatectomy (11 weeks after the initial X-ray exposure), a single terminal partial hepatectomy (17 weeks after initial X-ray exposure), 3 successive partial hepatectomies (11, 14, and 17 weeks after initial X-ray exposure), and a single hepatectomy 17 weeks after irradiation. These results, shown in table 2, contain no indication of any decrease in the frequency of abnormal mitoses after successive stimuli to regeneration. Figures 1 to 4 illustrate some typical nuclear abnormalities during various stages of division commonly observed in previously irradiated regenerating liver.

Relative Cell Size

The two sets of observations reported so far established a somewhat paradoxical situation for liver regeneration in the previously irradiated rat: The restoration of liver mass appeared to proceed undisturbed, even though the amplitude of the mitotic wave was greatly reduced in the irradiated subjects. To clarify further this situation, and to establish whether mass restoration in these livers did proceed in the face of a smaller total number of mitoses, relative to nonirradiated rats, an attempt was made to estimate relative cell sizes in these two types of regenerated livers. Tissues from Group II (text-fig. 1), subjected to histological processing under identical and closely controlled conditions, were selected for this purpose.

The results (table 3) failed to reveal differences in mean liver cell size between irradiated and control groups not subjected to partial hepatectomy. Based on the assumption (*see* "Methods") that the cell number represents the total cell population in a reproducible volume, the calculated ratio of cell volume of the proper controls to that of the irradiated livers after 2 and 3 partial hepatectomies is $\frac{(C_2)}{(R_2)} = 1.165$, and $\frac{(C_3)}{(R_3)} = 1.220$, respectively. If based on the assumption that the cell number

TABLE 3.—Parenchymal cells/100 high-power fields, as related to cell size*

Nonirradiated			X-irradiated		
C_1 Nonpartial hepatectomy	C_2 3 Weeks after second partial hepatectomy	C_3 3 Weeks after third partial hepatectomy	R_1 Nonpartial hepatectomy	R_2 3 Weeks after second partial hepatectomy†	R_3 3 Weeks after third partial hepatectomy‡
4820 ± 256	4350 ± 164	4662 ± 215	4540 ± 332	3730 ± 261	3825 ± 315

*All values reported are mean ± standard deviation.

†X irradiated, 2 partial hepatectomies $\frac{C_2}{R_2} = 1.165$, $\left(\frac{C_2}{R_2}\right)^{3/2} = 1.26$.

‡X irradiated, 3 partial hepatectomies $\frac{C_3}{R_3} = 1.220$ $\left(\frac{C_3}{R_3}\right)^{3/2} = 1.345$.

represents the cell population within a single plane cross-sectional area, the calculated ratio of cell volumes of proper controls to that of the irradiated livers after 2 and 3 partial hepatectomies is $\left(\frac{C_2}{R_2}\right)^{3/2} = 1.26$, and $\left(\frac{C_3}{R_3}\right)^{3/2} = 1.345$, respectively. The cell size of livers of irradiated animals subjected to 2 or 3 successive partial hepatectomies is thus distinctly larger than that of similarly treated controls ($P<0.01$).

DISCUSSION

Alterations in cell-population dynamics and cell function as a result of radiation damage to the liver-cell nuclei appear important with relation to the problem of delayed radiation effects. The normal adult liver, which is characterized by only a few mitoses per 10,000 parenchymal cells, can be stimulated by partial hepatectomy or some hepatotoxic agent to elicit an intense wave of mitoses resulting in complete or almost complete replacement of the removed tissue. The data show that in the liver subjected to irradiation there was 1) adequate restitution of the mass of the resected liver; 2) a significant decrease in the mitotic index, compared to nonirradiated controls; and 3) an occurrence of abnormalities in 80 percent of the mitotic figures observed in anaphase and telophase. There was no decrease in frequency of abnormal mitoses after repeated massive resections leading to extensive replacement of the original tissue mass. The failure of the incidence of mitotic abnormalities to decrease under these conditions indicates that the presumably uninjured cells (*i.e.*, without visible mitotic abnormalities) possess no competitive advantage over the injured cells in replacement of liver tissue, and that the mitotic abnormalities observed can be carried down to daughter cells at least one generation beyond the cells receiving the primary injury.

The question of the radioresistance of hepatic tissue has never been fully reconciled. Although previous reports have indicated that X-ray exposure of rats to doses in excess of 1000 to 2000 r does not affect the morphology of liver (*1*, *7*, *8*) nor the ability of liver to regenerate after partial hepatectomy (*7*), it is well established that similar exposure to ionizing radiation definitely decreases the mitotic index of proliferating tissue (*8–10*). In the present experiment, the level of radiation exposure (2 doses, 400/exposure), which is well below that used by other investigators, is injurious enough to demonstrate microscopic evidence of decreased mitotic index and increased nuclear damage of regenerating tissue. It is definitely suggested that this is a sensitive test of radiation damage if cell division is initiated after X-ray exposure.

The peak of mitotic frequency for the rat is usually marked somewhere between 28 and 32 hours (*3*) and declines rapidly thereafter to premitotic values within 7 days. In the animals subjected to 3 successive partial hepatectomies, the mitotic index of the irradiated animals was signifi-

cantly lower than the controls killed at 32 hours, whereas there was no significant difference for the irradiated rats and controls killed at 36 hours (table 1). This would suggest that the mitotic wave of the irradiated animals has been shifted so that the relative peak of mitoses is occurring later than the controls or else the subsequent decline of mitoses after the peak is much less for the irradiated tissue than for the controls. In line with this conclusion, Weinbren (*8*) has demonstrated, in far more severely irradiated liver tissue, that when cell proliferation is induced 2 months after X-ray exposure, the mitotic index remains higher in the irradiated specimens than in the controls after the peak has been attained.

In contrast to these histological findings, the irradiated animals are capable of restoring liver mass as readily as the controls, after partial hepatectomy. This is indicated by the same liver-weight/body-weight ratio 3 weeks after surgery for the irradiated and nonirradiated animals after a single or several repetitive partial hepatectomies. As an explanation for this paradoxical relationship of decreased mitotic index to the unaltered liver weight/body weight after exposure to X ray, the data (table 3) show that parenchymal cell size increases after cell division is initiated, *i.e.*, larger liver cells make up the restored liver mass. This observed increase in parenchymal cell size may bear some relation to the giant cell previously reported as occurring in irradiated mammalian cells (HeLa) that have received X-ray doses (*11*).

Our data confirm the findings of previous investigators (*1*, *2*, *8*, *12*) of the presence and persistence of abnormal mitoses in irradiated tissue when subjected to cell proliferation. Of even greater interest, however, at the radiation levels used in this experiment, is that the frequency of abnormal mitoses (80%) remains unchanged after successive stimuli to mitoses. The control experiments demonstrate that several weeks after each partial hepatectomy, prior to the next resection, there is no evidence of unusual mitotic activity in the irradiated animals. Thus, no part of the observed abnormal mitoses could conceivably be interpreted as a remnant from the previous regeneration episode, which thus falsifies the conclusion reached.

No effort has been made to differentiate the abnormal mitotic figures resulting from "physiological" or "structural" defects. Both the "physiological" defects, characterized by stickiness and clumping of the chromosomes at metaphase and formation of bridges at anaphase that result in incomplete splitting of the separate daughter nuclei, and the "structural" defects, characterized by chromosome breakage and fragmentation with particles scattered through the cytoplasm, were observed in our experiments. Presumably the so-called "physiological" defects (*13*) occur only in cells in active division at this time of irradiation, and thus they are transient and short lived. Since all the animals in the present study were subjected to X-ray exposure while the vast majority of the liver cells were in the nondividing stage (intermitotic), such specific nomenclature cannot be applied with any reasonable interpretation to the type of defect observed.

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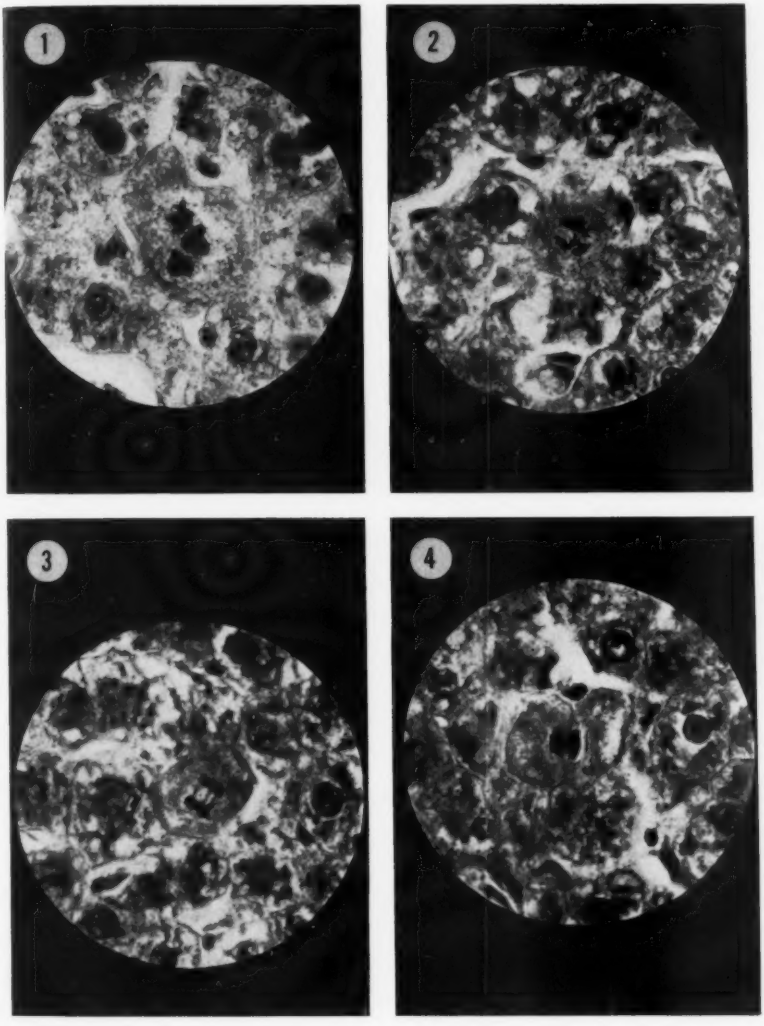
PLATE 22

FIGURE 1.—Tripolar formation, anaphase. × 500

FIGURE 2.—Fragmentation of chromatin material, anaphase. × 500

FIGURE 3.—Chromosome bridges, late anaphase. × 500

FIGURE 4.—Failure of daughter nuclei to split, telophase. × 500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Aminofluorenols as Urinary Metabolites of *N*-2-Fluorenylacetamide [1]

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SUMMARY

The isomeric hydroxylated derivatives of 2-fluorenamine, *i.e.*, the 2-amino-1-, -3-, -5-, -7-, and -8-fluorenols, were found in small amounts as free compounds and as glucuronic acid conjugates in the urine of rats given *N*-2-fluorenylacetamide. The presence of the metabolites was established by specially designed solvent partition steps followed by chromatography and specific isotope-dilution procedures. The aminofluorenols could be the result of the hydroxylation of 2-fluorenamine or the deacetylation of the corresponding *N*-(hydroxy-2-fluorenyl) acetamides which are known metabolites of *N*-2-fluorenylacetamide.—J. Nat. Cancer Inst. 27: 145–152, 1961.

BIOCHEMICAL HYDROXYLATION in rats converts the carcinogen 2-fluorenylacetamide to isomeric derivatives bearing hydroxyl groups on carbons 1, 3, 5, 6, 7, and 8, and retaining the acetyl group (*1–4*). The occurrence of deacetylation (*cf. 1*) might suggest that the corresponding aminofluorenols would also be found as metabolites. This paper deals with the demonstration, by chromatography and the specific inverse isotope-dilution technique, that aminofluorenols are, indeed, excreted in the urine by rats given *N*-2-fluorenylacetamide.

MATERIALS AND METHODS

Female Buffalo strain rats [4] weighing about 170 g were given an oral dose of 10 mg/100 g body weight of 9-C^{14}-labeled *N*-2-fluorenylacetamide [specific radioactivity, 1.68×10^6 counts per minute (cpm)]. The urine was collected in ice-cold receivers for 48 hours (*2*).

Chromatographic demonstration of aminofluorenols.—The urine (13 ml, 12.3×10^6 cpm) was buffered at *p*H 6 with 1 M acetate buffer (1 ml) and incubated overnight at 37° C with bacterial β-glucuronidase and Taka-

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[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.
[4] Supplied by Dr. Harold P. Morris.

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diastase. The mixture was then boiled for ½ minute, cooled, and centrifuged. The pellet was extracted with 1 ml of hot ethanol and then 10 ml of water; the supernatants comprising the urine and washes after centrifugation were combined and adjusted to 25 ml with water. The solution was extracted 5 times with 25 ml portions of freshly distilled ether. The combined ether extracts were washed with 5 ml of water, which was added to the aqueous phase (step *A*, table 1).

The ether layer was extracted with a solution containing 0.5 mg each of 2-amino-1-, -3-, -5-, and -7-fluorenol in 100 ml of 0.1 N hydrochloric acid, 100 ml of the acid alone, and a little water (step *B*) in that order. The aqueous, acid layer was re-extracted with 50 ml of ether to remove any acetylamino derivatives present by virtue of a favorable partition ratio (step *C*).

The acid layer[5] containing the aminofluorenols was carefully neutralized to *p*H 7 with solid sodium bicarbonate, extracted 8 times with 50 ml of ether (step *D*, 4 times; step *E*, 4 times).

Suitable aliquots of all solutions obtained during these partitions were chromatographed on paper in cyclohexane, *tert.*-butanol, acetic acid, and water (16:4:2:1). The paper chromatograms were exposed to film as described (*5*).

General procedure for inverse isotope dilution.—To a solution of 250 to 350 mg of the aminofluorenol in 50 ml of 0.12 N hydrochloric acid, we added 1.5 to 2.5 ml of urine containing 1 to 2 $\times$ 10^6 cpm for determination of free compounds, or of glucuronidase-incubated urine for determination of free compounds plus glucuronic acid conjugates. The filtered solution was neutralized with 0.5 g of sodium bicarbonate. The resulting solid was dissolved in dilute acid and reprecipitated by bicarbonate. A solution of the material in 50 ml of 0.12 N hydrochloric acid was extracted 3 times with 20 ml portions of freshly distilled ether for removal of neutral or acidic metabolites. The aminofluorenol was then reisolated by addition of bicarbonate and purified by repeated crystallizations. For this purpose the compound was usually dissolved in 20 to 50 ml of ethanol, and 50 to 100 ml of boiling water was slowly added. The solution was filtered and evaporated slightly. Upon cooling, the aminofluorenols crystallized as white or slightly tan needles. When the specific radioactivity was constant as determined after each step, the aminofluorenol was acetylated and the acetyl derivative crystallized once or twice from dilute acetic acid or ethanol. Reconversion to the aminofluorenol by refluxing in 3 N hydrochloric acid and crystallization confirmed the constancy of the specific activity that was used in the calculations (*6*).

A control experiment to test these procedures involved the mixing of 329.7 mg of inactive, carrier 7-amino-2-fluorenol and 1.92 mg of biosynthetically obtained 9-C^{14} *N*-(7-hydroxy-2-fluorenyl) acetamide, a closely related material. The aminofluorenol obtained after the first 2 operations showed a specific radioactivity of 715 cpm/mg. However,

[5] Pilot experiments with 2-fluorenamine and 7-amino-4-fluorenol(2-amino-5-fluorenol) showed that these typical amines were present almost totally in the acid extract.

after a series of further purification steps similar to those involving the aminofluorenols and labeled urine, the specific activity of the carrier compound dropped to zero. Thus, the methodology employed was adequate for the separation of structurally similar yet different compounds.

RESULTS

Chromatographic Studies

Hydrolysis by β-glucuronidase and Taka-diastase of the urine of 2 rats fed labeled *N*-2-fluorenylacetamide, followed by ether extraction, resulted in the transfer of 44 percent of the dose to the organic layer of the urinary radioactivity (step *A*, table 1). This ether extract was expected to contain most of the hydroxylated metabolites, such as the *N*-(hydroxy-2-fluorenyl)acetamides and aminofluorenols. The free amino group in the latter class of compounds was separated from the other metabolites by transfer into dilute hydrochloric acid (step *B*). Small amounts of some unlabeled aminofluorenols (*cf.* "Methods") were added at this point to act as carriers. Without this precaution the trace quantities of the radioactive aminofluorenols present could not be demonstrated readily and unambiguously, because of losses due to oxidation and also as a result of solubility in both layers during the partition process. The acid, aqueous phase accounted for 6.1 percent of the dose. However, because of the appreciable water solubility of hydroxylated metabolites other than aminofluorenols, an extraction with ether was performed (step *C*). This left 3 percent in the aqueous layer. Neutralization, followed by extraction with ether, transferred the aminofluorenols to the organic phase (steps *D* and *E*). Repeated extractions were required. In all, 2.3 percent of the dose was accounted for by the combined organic phases from these extractions, which would correspond to metabolites bearing a basic substituent including the aminofluorenols.

TABLE 1.—Extraction of aminofluorenols from urine*

Solution extracted	Percent of dose †	
	Ether phase	Aqueous phase
Hydrolyzed urine by ether: *A*	44	16
Ether *A* by 0.1 N HCl: *B*	37	6.1
Aqueous *B* by ether: *C*	2.9	3.0
Neutralized aqueous *B* by ether: *D*	1.9	1.0
Aqueous *D* by ether: *E*	0.4	0.6

*The pooled 48-hour urine from 2 rats accounted for 64 percent of the dose (measured as radioactivity) of 9-C^{14}-labeled *N*-2-fluorenylacetamide (*see* text).

†Calculated from the partition of the urinary radioactivity under the conditions listed.

Chromatography of the ether extracts obtained as described yielded the results illustrated in table 2. The autoradiographs of chromatograms

of fraction *A* showed darkening of the film mainly in the areas corresponding to the mobilities of the known hydroxylated derivatives of *N*-2-fluorenylacetamide. In addition, however, there was some general darkening of the film between spots, ascribable to small amounts of metabolites with intermediate mobility. Fraction *B*, freed of the basic metabolites, appeared to be much cleaner and showed mainly the spots due to the various *N*-(hydroxy-2-fluorenyl)acetamides (*5*). Fraction *C*, the wash of the aqueous phase, contained chiefly the 7- and 5-hydroxy derivatives, some of which, being more polar, might well have transferred to the aqueous phase during step *B*.

TABLE 2.—Chromatography of ether extracts

	Fraction (from table 1)				
	A	*B*	*C*	*D*	*E*
Compounds*	R_f values × 100 and amounts †				
7-Amino-2- and -4-fluorenol	—	—	—	0–3 (11. 4)	0–4 (15. 4)
7-OH-2-FAA	2–8 (8. 3)	3–9 (7. 6)	1–10 (15. 3)	—	—
2-Amino-1- and -3-fluorenol	—	—	—	3–12 (8. 0)	4–14 (7. 5)
5-OH-2-FAA	8–18 (9. 3)	11–20 (7. 3)	10–19 (7. 3)		
				12–19 (4. 1)	14–18 (1. 3)
				24–34 (1. 2)	24–38 (1. 3)
2-Fluorenamine				38–50 (0. 4)	
3-OH-2-FAA	33–47 (2. 2)	34–48 (2. 6)			
					42–62 (0. 8)
N-2-Fluorenylacetamide 1-OH-2-FAA	58–83 (3. 2)	55–83 (3. 5)			

*Nomenclature and abbreviations: 7-amino-2-fluorenol = 2-amino-7-fluorenol; 7-amino-4-fluorenol = 2-amino-5-fluorenol; 7-OH-2-FAA = *N*-(7-hydroxy-2-fluorenyl)acetamide, and similarly for the isomeric compounds.

† Relative amounts are given in parentheses under each R_f value. They are expressed in mμmoles of *N*-2-fluorenylacetamide, with a conversion factor of 1 mμmole = 375 cpm. The results are presented in terms of 26.7 mμmoles of compound or 10,000 cpm applied per strip. The difference between 26.7 mμmoles and the sum of the amounts attributed to each spot on a given strip represents nonlocalized radioactivity between spots. We determined the data by cutting the paper chromatograms carefully according to the spots seen on the autoradiographs. The individual paper spots were counted in a Packard Tri-Carb scintillation spectrometer. The solvent used was composed of cyclohexane, *tert.*-butanol, acetic acid, and water (16:4:2:1), and the paper was Schleicher and Schuell No. 598, cut in strips 7 mm wide (*5*).

The chromatograms of fractions *D* and *E* showed the spots due to the aminofluorenols. Of these, the slow-moving area near the origin was characteristic of 7-amino-2- and -4-fluorenol. The spot was very dense and tight in contrast to some of the faster-moving ones that were more elongated and appeared less intense. Those with mobilities of 0.03 to 0.12 had an R_f value like that of 2-amino-1- and -3-fluorenol. The adjoining area, listed as a distinct spot, might likewise be due to these compounds, but could also represent another material. The transition between these 2 areas was diffuse. The spot with a mobility of 0.24 to 0.34 was

encountered occasionally in chromatograms of ether extracts of urines (*7*, *8*) and also of hydrolysates of liver proteins from rats given *N*-2-fluorenylacetamide (*9*). In view of its appearance on the chromatograms of the compounds with a basic substituent, this as yet unknown material might have a free amino or some similar group. A trace of a substance with the mobility of 2-fluorenamine was noted on the first extract *D*, but not on extract *E*, which contained, however, a small amount of a material with the mobility of 0.42 to 0.62.

Isotope-Dilution Experiments

The isotope-dilution studies showed that the urine of rats given a single dose of *N*-2-fluorenylacetamide contained only small quantities of the isomeric aminofluorenols (table 3). The compound present in largest amount was 7-amino-4-fluorenol, but it accounted for only 1.5 percent of the urinary radioactivity. All the other 2-aminofluorenols studied,[6] namely the compounds hydroxylated at the 1, 3, 7, and 8 positions, corresponded to 0.1 to 0.3 percent, as the unconjugated compounds. Larger proportions of the aminofluorenols were found largely in the free state, and smaller ones as glucuronic acid conjugates. In contrast, the acetyl derivatives were observed mostly as conjugates (*1*).

TABLE 3.—Percentage of aminofluorenols in urine as determined by inverse carrier isotope dilution *

Compound	Mg of compound	Specific activity in cpm/mg first and last 3 determinations	C^{14} in urine sample cpm $\times 10^{-4}$	Percent of urinary C^{14}
2-Amino-1-fluorenol	217	81, 23, 24, 24	152	0.3
2-Amino-1-fluorenol glucuronide†	231	79, 12, 14, 10	171	0.2
2-Amino-3-fluorenol	208	245, 32, 35, 35	152	0.5
2-Amino-3-fluorenol glucuronide†	263	126, 34, 34, 33	172	0.1
7-Amino-1-fluorenol‡	242	61, 12, 9, 10	152	0.2
7-Amino-1-fluorenol glucuronide†	343	44, 17, 19, 16	172	0.2
7-Amino-2-fluorenol§	207	39, 3, 3, 3	113	0.1
7-Amino-2-fluorenol glucuronide†	204	111, 16, 18, 16	113	0.3
7-Amino-4-fluorenol‖	244	151, 108, 103, 99	152	1.5
7-Amino-4-fluorenol glucuronide†	315	294, 120, 110, 111	172	0.5

* Data obtained in one experiment involving the pooled urine of 4 female Buffalo strain rats given 10 mg/100 g *N*-2-fluorenylacetamide. Two earlier pilot experiments with 7-amino-2-fluorenol performed under less rigorous conditions (without extraction of the acid solutions with ether to remove the acetylamino derivatives) gave somewhat higher values, 1.2 and 2.5 percent, for the free compound. The experiments listed above usually involved 15 to 20 distinct operations for the purification of the carrier compound. The specific radioactivity of the carrier is given only after the first and last three operations.

† Glucuronides were determined by the difference in activity accounted for by the carrier compound before and after hydrolysis of the sample of urine with bacterial β-glucuronidase.

‡ *Chemical Abstracts'* nomenclature; common name, 2-amino-8-fluorenol.

§ Or 2-amino-7-fluorenol.

‖ Or 2-amino-5-fluorenol.

[6] It seems probable that the other isomeric aminofluorenols would likewise occur as urinary metabolites of *N*-2-fluorenylacetamide. However, experiments to substantiate this were not performed.

DISCUSSION

With the demonstration that small proportions of a dose of *N*-2-fluorenylacetamide are excreted in the urine as various isomeric aminofluorenols, the question as to their mode of formation arises. These compounds could be produced by the deacetylation of the corresponding acetyl derivatives, *i.e.*, the *N*-(hydroxy-2-fluorenyl)acetamides, which are known metabolites. Alternatively, direct hydroxylation of fluorenamine, also a metabolite of *N*-2-fluorenylacetamide, would result in the aminofluorenols. Actually, there is some evidence that both modes may be operative. Thus, Miller *et al.* (*8*), reporting on the metabolism of acetyl-labeled *N*-2-fluorenylacetamide, noted that the various hydroxylated metabolites had lost about one half of the label, except for the 1-hydroxy derivative, which had specific activity corresponding to an exchange of two thirds of the isotope. Furthermore, according to Gutmann *et al.* (*10*), many rat tissues deacetylate *N*-2-fluorenylacetamide and the 1-hydroxy derivative, but only a few tissues deacetylate the 7- and 5-hydroxy derivatives.

These two sets of experiments suggest that the *ortho*-aminohydroxy compounds could be produced by deacetylation in many tissues, but the other aminofluorenols only in such tissues as the kidney, adrenal glands, and brain. In this connection, the deacylase activity of the kidney seems particularly pertinent, since the aminofluorenols, which are sensitive to oxidizing conditions, could appear in the urine without undergoing oxidative decomposition. The urine is rather low in oxidative systems. Yet, while transport by the blood would expose the aminofluorenols to a more oxidizing environment, it is conceivable that these compounds exist in the blood in some loosely bound form which would tend to stabilize them. In any case, these data are consistent with the concept that aminofluorenols arise both by direct hydroxylation of 2-fluorenamine and by deacylation of the various *N*-(hydroxy-2-fluorenyl)-acetamides.

A related study was performed to test the idea that 2-fluorenamine might yield relatively larger amounts of *ortho*-hydroxylated metabolites than would *N*-2-fluorenylacetamide. In the comparable case of 2-naphthylamine, the administration of the free amine gave larger amounts of *ortho*-hydroxylated products than the acetyl derivative, which led mainly to the 6-hydroxy compound (*11*, *12*). However, we found that the paper chromatograms of urines and urine extracts of rats given an oral dose of C^{14}-labeled 2-fluorenamine were essentially similar to those obtained with *N*-2-fluorenylacetamide (*5*), an observation also made by Miller *et al.* (*8*) with unlabeled 2-fluorenamine. The conclusion must be drawn that in rats the equilibrium of the reversible conversion *in vivo* between 2-fluorenamine and *N*-2-fluorenylacetamide lies on the side of the latter type of compound. Thus, the bulk of the metabolites is found in the urine as the acetyl derivative.

Another question deals with the significance of the aminofluorenols in the carcinogenic process and the phenomena related thereto. Binding

of metabolites of the carcinogen to tissue constituents may be in some way involved in, or indeed may be essential to, the carcinogenic process (*1*). Gutmann and associates (*10*) in their more recent publications have postulated that the deacetylated products, especially 2-amino-1-fluorenol, are the essential intermediates capable of binding to tissue constituents after enzymic oxidation to the reactive quinone imines. We have shown, furthermore, that the free aminofluorenols give rise to protein-bound radioactivity as an artifact during homogenization of liver with these compounds (*9*). Moreover, oxidation of these compounds can result in insoluble polymers simulating protein-bound materials, as discussed in an earlier paper (*1*). In the course of studies (*9*) on the enzymic hydrolysis of liver proteins with bound radioactivity from *N*-2-fluorenylacetamide, we have occasionally found undigested, insoluble residues with a higher specific radioactivity than the starting protein. This residue could have been such a polymer, though the specific activity was not as high as that of the carcinogen administered. In any case, while the aminofluorenols no doubt give rise to protein- or tissue-bound metabolites, the possibility of their involvement in the carcinogenic process has been weakened to some extent by the discovery by Miller and associates (*13*) of *N*-hydroxylation of *N*-2-fluorenylacetamide. Since the resulting *N*-hydroxy-*N*-2-fluorenylacetamide appears to be more active than *N*-2-fluorenylacetamide, especially in regard to the production of sarcomas and other local tumors at the point of application (*8*, *14*), it may be close to, if not actually the "proximate" carcinogen. Of course, deacylation could occur even in this case, so that the effective compound might really be *N*-2-fluorenylhydroxylamine.

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On the *In Vitro* and *In Vivo* Protein-binding of *N*-2-Fluorenylacetamide and Related Compounds [1]

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SUMMARY

Data are presented on the distribution of radioactivity after one intraperitoneal injection of C^{14}-labeled *N*-2-fluorenylacetamide (2-FAA) into rats as a function of dose, from 10 to 0.01 mg/100 g, and of time, from 6 hours to 32 days. The urinary metabolites were qualitatively and quantitatively similar at all time intervals and dosages. The activity in blood, liver, and soluble and insoluble liver proteins decreased with decreasing doses, but not directly proportionally. The isotope in blood and liver, highest at 6 hours, dropped more rapidly in liver than in blood. Maximal labeling of the liver proteins occurred at 1 day. Hydrolysis of the labeled proteins by acid, alkali, or enzymes rendered 50 to 60 percent of the activity extractable into organic solvents. Details of the solvent partition and chromatographic experiments performed on the hydrolysate are presented. Amounts of 0.3 and 0.7 percent of the activity in the labeled protein were due to 2-FAA and 2-fluorenamine, respectively. The balance consisted of at least 6 more polar materials. Thus, with 2-FAA more than one metabolite and protein are involved in the labeling of tissues. Homogenization of rat liver with labeled 2-FAA or hydroxylated metabolites under various conditions gave negligible binding. However, the aminofluorenols studied led to considerable "protein-bound" activity by homogenization or by the mixture of soluble proteins with a solution of the compound. This artifact-binding took place to a larger extent with the *ortho*-aminofluorenols than with 7-amino-2-fluorenol. In contrast to the binding *in vivo*, no radioactivity was rendered extractable into organic solvents after hydrolysis of the proteins labeled *in vitro*. Artifact-binding seems to play only a limited role under conditions *in vivo*.—J. Nat. Cancer Inst. 27: 153-171, 1961.

INITIATION OF the carcinogenic process by chemicals may involve the combination of the compound, or a metabolite thereof, with the tissue affected. This has been a useful working hypothesis ever since the first experimental demonstration of the phenomenon with the azo dyes by the Millers (*1*). Numerous other carcinogens have been examined for their ability to combine with tissue constituents since that time, and thus far no

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[2] With the expert technical assistance of Mrs. Anna R. Parker.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

exceptions have been observed. Hence, binding of carcinogen to tissue may be a necessary, although not sufficient, prerequisite for carcinogenesis (*2*). With the potent carcinogen *N*-2-fluorenylacetamide (2-FAA, 2-acetylaminofluorene), the binding of isotope derived from the labeled compound to the proteins of a variety of tissues was reported first by Dyer,[4] Miller and Miller (*4*), and Weisburger *et al.* (*5*, *cf.* *4*), and subsequently by Gutmann *et al.* (*6*), Sorof *et al.* (*7*), and Hultin (*8*, *9*—*see also* *10*). To elucidate further the interaction of carcinogen and tissue, we undertook a series of pertinent experiments.

This paper reports data on protein-bound radioactivity, as a function of dose administered and as a function of time in the livers of rats, after an intraperitoneal injection of C^{14}-labeled 2-FAA. Attempts to identify the bound metabolites by hydrolysis of the proteins and chromatography of the hydrolysate extracts are described. We also studied the possible formation of protein-bound radioactivity as an artifact during the homogenization of tissue containing radioactive metabolites of the carcinogen. The metabolites present in the urine in largest amounts, *i.e.*, *N*-(hydroxy-2-fluorenyl)acetamides and conjugates, did not produce such artifacts. However, the free aminofluorenols led to protein-bound radioactivity by homogenization of the compound and liver or when the soluble liver proteins and the compound were mixed.

MATERIALS AND METHODS

Experiments In Vivo

Effect of a single intraperitoneal dose on liver protein-bound activity.—Seven pairs of 150 g female Buffalo strain rats [5] received intraperitoneal injections of an acacia suspension of 9–C^{14}-labeled 2-FAA [6] (*11*). The first 2 pairs received 10 and 5 mg, respectively, per 100 g body weight of compound with a specific activity of 1.68×10^6 counts per minute (cpm)/mg. The next 5 pairs were given 1, 0.5, 0.1, 0.05, and 0.01 mg, respectively, per 100 g body weight, of compound with a specific activity of 3×10^7 cpm/mg. Each pair of rats was kept in a glass metabolism cage. Urine was collected in an ice-cold receiver. Purina laboratory chow and water were available *ad libitum*. After 24 hours the animals were killed by decapitation. Aliquots of blood were collected in heparinized vessels.

After perfusion with cold saline, the livers from each pair of animals were pooled, and an aliquot weighing 4 g was homogenized (motor-driven teflon pestle in a glass tube) for 2 minutes in 40 ml of 0.1 M acetate buffer,

[4] Dyer observed binding of isotopic nitrogen to the soluble and insoluble proteins of livers of rats intubated with N^{15}-2-FAA, as reported at a staff meeting, Laboratory of Biochemistry, National Cancer Institute, March, 1952 (*3*).

[5] Supplied by Dr. Harold P. Morris.

[6] Intraperitoneal injections of a similar gum-acacia suspension of 2-FAA, given 3 times weekly for several months, induced tumors of the mammary gland, liver, and ear duct in rats (unpublished observations of H. P. Morris and coworkers).

*p*H 5. After centrifugation at 950 × *g* for 20 minutes, the pellet was resuspended in an equal volume of buffer, stirred at intervals for 1 hour, and centrifuged again. The pellet, labeled "insoluble protein," was washed with ethanol, extracted with this solvent in a Soxhlet apparatus for 40 hours, and air-dried. The combined aqueous supernatants and washings were heated for 10 minutes on a steam bath. The resulting protein precipitate, called "soluble protein," was likewise subjected to ethanol extraction.

The urines and samples of urines hydrolyzed with β-glucuronidase and with Taka-diastase were extracted with ether. Aliquots of the extracts were chromatographed in 2 solvent systems, *sec.*-butanol—3 percent NH_4OH (3:1), and cyclohexane, *tert.*-butanol, acetic acid, water (16:4:2:1), as reported previously (*12*).

Effect of time after a single dose on liver protein-bound activity.—Eight pairs of 150 g rats received injections of 5 mg of labeled 2-FAA and were placed in metabolism cages. The first 5 groups received the compound with the lower specific activity, the last 3, the one with the higher activity. One pair of rats was killed after 6 and 12 hours, and 1, 2, 4, 8, 16, and 32 days, respectively, and treated as described for the preceding experiment.

Experiments In Vitro

Hydrolysis of liver proteins; extraction and chromatography of hydrolysate.—Amounts of 100 to 700 mg of dry protein were refluxed in 10 to 25 ml of 6 N hydrochloric or sulfuric acid, 5 N NaOH, or saturated barium hydroxide solution for 18 hours. The ice-cooled solutions were carefully neutralized with concentrated NH_4OH (acid hydrolyses) or dry ice followed by acetic acid (alkaline hydrolyses) to a final *p*H of about 6. The solutions were treated with acetic anhydride to acetylate any freed metabolite (*cf. 10*). After addition of 50 percent saturated $(NH_4)_2SO_4$ solution the acetylated hydrolysates were extracted with ether-ethanol (3:1 parts). Aliquots of the extracts were chromatographed on paper (*12*).

Alternatively the proteins (in 140–180 ml of buffer/g) were incubated (38°) for 2 to 3 days with pepsin (twice recrystallized, Nutritional Biochemical Corp., Cleveland, Ohio, about 300 mg/g protein) at *p*H 2 in the presence of a few drops of chloroform and toluene, followed by 2 to 3 days with trypsin (twice recrystallized, Nutritional Biochemical Corp., about 150–200 mg/g protein) at *p*H 8. The latter step was usually repeated after the addition of more trypsin. Other hydrolyses were similarly performed with pancreatin (100–200 mg/g protein) at *p*H 8 over 8 to 12 days, or with Hydrolase Mixture [7] (100–200 mg/g protein of the enzyme concentrate from *Aspergillus oryzae*, supplied by C. F. Boehringer, Mannheim, Germany) at *p*H 7 over 6 to 12 days. Fresh enzyme preparations were added to these every 2 to 3 days. In general,

[7] We are indebted to Dr. C. Heidelberger, University of Wisconsin, Madison, Wis., for suggesting this preparation.

the solid proteins went into solution within 1 to 2 days; the "soluble liver protein" fraction dissolved in a shorter time than the "insoluble" fraction.

The incubation mixtures were adjusted to *p*H 7 and three extractions with equal volumes of ether were carried out. The aqueous phase was further extracted with ether-ethanol (3:1), then with *n*-butanol. The solution was sometimes saturated 50 percent with $(NH_4)_2SO_4$ and extracted with ether-ethanol (3:1). In a few instances the aqueous phase remaining after those partitions was freed of organic solvent by ether extraction and aeration and then subjected to acetylation with acetic anhydride at *p*H 7. Further treatment with ether, ether-ethanol, and *n*-butanol effected the removal of materials then soluble in the organic phase. Alternately, the aqueous phase was dialyzed against distilled water, and the dialysates were subjected to the sequence of solvents. Aliquots of all samples were chromatographed on paper or on silicic acid columns (*12*, *13*). In one instance the presence of 2-fluorenamine and 2-FAA in the appropriate chromatographic fraction was ascertained by the inverse isotope-dilution technique as practiced heretofore with fractions of the corresponding urinary metabolites (*13*).

Study of artifact binding during homogenization.—The ice-cold, perfused livers from 200 g female Buffalo strain rats were coarsely minced with scissors and divided into 1.5 g portions. Each portion was homogenized with 1.5 mg of the C^{14}-labeled compound (listed in table 5) in 6 ml of 0.2 M acetate buffer, *p*H 5, under the following conditions: 1) In a Waring blendor with an ice-cold, stainless-steel, 50 ml microhead operated at full speed for 2 minutes. The homogenate was separated into a soluble protein and an insoluble protein fraction, as described for the studies *in vivo*. These fractions will be labeled W_s and W_i, respectively. 2) In the Waring blendor, but after the scissor-minced liver portion had been heated for 10 minutes on a steam bath in the buffer solution. The resulting protein will be called W_h. 3) In a Potter-Elvehjem-type homogenizer with a teflon pestle in an ice bath for 3 minutes, and then separation of a soluble, P_s, and insoluble, P_i, protein fraction. 4) In a Potter-Elvehjem homogenizer, but with heated liver, P_h. Thus, 6 different protein fractions were studied with each compound. The homogenates were treated as described for the experiments *in vivo*, which yielded the ethanol-extracted dry proteins.

In another experiment, the soluble protein fraction from 11.4 g of liver was isolated. The ice-cold solution (100 ml) was divided into 2 equal parts, and air and nitrogen, respectively, were bubbled through each for 15 minutes. Four 12 ml aliquots of each protein solution were added to tubes containing C^{14}-labeled 7-amino-2-fluorenol (2-amino-7-fluorenol) from 1.5 mg of the acetyl derivative, prepared as described later. The well-stoppered tubes were stored in an ice bath. After 0, 1, and 24 hours, one tube from the air- and one from the nitrogen-blown solutions were heated for 10 minutes on a steam bath, and the coagulated proteins were isolated, washed, and extracted with ethanol.

The C^{14}-labeled derivatives of 2-FAA were prepared biosynthetically as described for *N*-(1-, 3-, and 7-hydroxy-2-fluorenyl)acetamide (*11*, *13*). Thus the appropriate fractions obtained in the chromatography of the metabolites of 2-FAA on a silicic acid column (*14*) were diluted with the corresponding unlabeled carrier compounds. Each of these was then recrystallized to constant specific radioactivity (table 5).

The labeled aminofluorenols were prepared just prior to their use in the binding experiments, as follows: 4.4 mg of the radioactive *N*-(1-, 3-, or 7-hydroxy-2-fluorenyl)acetamide was refluxed with 6 ml of 1 N hydrochloric acid for 1 hour. Pilot studies with unlabeled compounds showed that hydrolysis was complete and gave the corresponding aminofluorenols with the correct ultraviolet spectrum. Upon cooling, the volume of the solution was 5.3 ml, of which two 25 μl aliquots were counted. An amount of 1.2 ml, equivalent to 1 mg of original compound, was added to each homogenizer tube, or the blendor container, and neutralized with 126 mg of solid sodium bicarbonate. Portions of 1 g of liver were used in these binding studies.

Determination of radioactivity in various samples.—The C^{14}-content of liquid samples such as urines, tissue homogenates, or solutions was determined in a windowless, gas-flow counter with a 48 percent efficiency, after aliquots were plated as infinitely thin layers on 1¾-inch-diameter glass discs. Dry solids such as feces or proteins were dissolved in 90 percent formic acid. For this purpose a 5 or 10 mg sample was accurately weighed into a 1 or 2 ml volumetric flask, respectively, and made to volume with formic acid. Most materials dissolved rapidly, but some, such as coagulated "soluble" rat liver proteins, required a 1-day incubation at 37° C or about ½ hour at 90° C (steam bath). Aliquots of 10 to 100 μl, depending on the amount of radioactivity, were plated. Samples of moderate to high specific activity were satisfactorily counted in this manner. However, materials with a low isotope content were combusted, and the radioactivity of the resulting barium carbonate was determined (*15*). Conversion factors for the 2 modes of sample preparation and other pertinent corrections were applied.

RESULTS

Effect of Dose and Time on Distribution of Metabolites

The doses selected for this experiment started with 10 mg/100 g of body weight and decreased to 0.01 mg in 6 steps, each involving 2 animals. For doses of 1 mg/100 g and lower, labeled 2-FAA of high specific activity (3×10^7 cpm/mg) was used to ensure the accurate measurement of small amounts of the compound and its metabolites by radioactivity determinations. Thus, the low blood level of metabolites represented by 0.13 mμmoles/ml of 2-FAA after the 0.01 mg/100 g dose (table 1) actually was derived by counting 50 μl aliquots of blood at necropsy. In this

TABLE 1.—Distribution of radioactivity as a function of dose 24 hours after a single intraperitoneal injection of *N*-2-fluoren-9-C^{14}-ylacetamide

Dose		Blood		Soluble liver protein*	Insoluble liver protein*	Urine	Percent of urine		
mg/100 g	cpm × 10^{-6}	(mμmoles/ml)	Liver*	(mμmoles/g)	(mμmoles/g)	(% of dose)	Unconjugated metabolites	Glucuronides	Sulfates
10	25	107	99(45%)	270	242	60	9	53	4
5	12.5	38	67(63%)	233	238	50	7	50	4.5
1	45	5.4	21(65%)	85	74	57	9	53	7
0.5	22.5	2.5	11(58%)	52	32	63	7	57	5.5
0.1	4.5	0.76	4.5(59%)	19	13.5	70	9	48	4.7
0.05	2.3	0.36	1.7(73%)	7.5	6.3	60	10	44	2.3
0.01	1.1	0.13	0.4(110%)	4.7	2.7	29	12	43	5.3

*The data are relative to wet weights for liver and dry weights for protein. An average of 0.2 g of dry soluble protein and 0.5 g of dry insoluble protein was obtained from 4 g of liver (wet weight). The percentage of the liver activity bound to proteins is given in parentheses.

case duplicate samples had net count rates of 41 and 38, or an average of 40 cpm on a counter with a background rate of 35 cpm. Thus, the gross count rate was more than twice the background rate. This example typifies some of the lowest counting rates in this study. Therefore, even the low values of radioactivity calculated and expressed in the tables as equivalents of mμmoles of 2-FAA are quite reliable.

A proportionality existed 24 hours after a single intraperitoneal dose between the amount of radioactivity in *blood* and *liver* (wet weight) and the amount of compound injected (table 1). The relationship was not quite linear, but the discrepancy from linearity was relatively small. The decrease in isotope concentration with dose was more pronounced from 10 to 1 mg than from 1 to 0.01 mg for blood, whereas for liver it was reversed. With the largest dose used, 10 mg, some material remained unabsorbed from the peritoneal cavity at 24 hours, as determined visually and by the activity of the white particles of compound revealed with a counter. This may be the reason for the similarity of the isotope levels bound to the soluble and insoluble *liver proteins* (dry weights) with the doses of 5 and 10 mg/100 g. The binding centers may have been saturated with the higher amount, though the total quantity of radioactivity in the blood and also in wet liver had not reached a limiting point. Below doses of 5 mg the specific activity of the liver proteins decreased with the amount of compound injected. However, a relatively constant amount of about two thirds of the total liver activity was bound to the proteins at the 1-day experimental period with doses below the 5 mg level. With the smallest dose of 0.01 mg all the radioactivity in the liver was apparently protein-bound.

The percentage of the dose excreted in the *urine*, about 60 percent, was largely independent of the amount injected (table 1). The smaller recovery on the lowest level of compound may be ascribed to loss by adsorption on spilled food, which can be relatively important with the minute masses involved. The quantity of unconjugated and conjugated metabolites remained relatively constant. Paper chromatography of all these samples revealed the same metabolites (*12*, *13*) in roughly similar amounts irrespective of the dose administered, as judged by the relative density of spots on the autoradiographs.

In the study of the *effect of time* on the distribution of isotope after an intraperitoneal dose, the animals examined at intervals up to 4 days received the labeled carcinogen with the lower specific activity. Those at the later periods received injections of the compound of higher specific activity, so that the accurate measurement of the small amounts of material expected at that time might be ensured.

The quantity of isotope excreted in the *urine* and *feces* was similar to that reported in earlier, less prolonged studies (*10*). Most of the radioactivity in the urine had appeared in 1 to 2 days; relatively minor amounts were eliminated in the later periods (table 2). Thus, on the 32d day, only 21,000 cpm were contained in the 22 ml sample of urine collected from the 2 animals which had received 300 million cpm. Ether extrac-

TABLE 2.—Distribution of radioactivity as function of time after a single intraperitoneal injection of 5 mg of *N*-2-fluoren-9-C^{14}-ylacetamide

Time (days)	Dose* (cpm $\times 10^{-6}$)	Blood (mμ moles/ml)	Liver	Soluble liver proteins †	Insoluble liver proteins †	Urine ‡		Feces, percent of dose
			(mμmoles/g)			Percent of dose	mμmole/ml	
0. 25	8. 4	38	238(12%)	162	175	20	460	—
0. 50	8. 4	27	81(44%)	264	184	57	1220	1. 7
1	8. 4	24	58(70%)	278	210	77	1240	8
2	8. 4	27	36(96%)	242	161	63	406	16
4	8. 4	19	21(100%)	224	108	71	9. 5	30
8	150	9. 9	5. 8(100%)	45	22	59	2. 3	22
16	150	8. 5	1. 4(87%)	7. 2	6. 3	69	1. 0	20
32	150	5. 4	0. 4(50%)	2	0. 8	77	0. 1	20

*Per rat.

† The data are relative to wet weight for liver, and dry weights for proteins. The percentage of the liver activity bound to proteins is given in parentheses.

‡ The specific activity in mμmoles/ml is shown for the urine collected over the entire period up to 2 days, and for the last 2 days of collection in the later periods. These data give a picture of the specific activity in the urine in relation to the activity in blood and tissues at the time of autopsy (*see* "Discussion").

tion of this urine followed by chromatography of the extracts showed similar patterns of metabolites as in urine obtained at the 1- and 2-day periods (*12*), though not all the spots on the chromatograms, especially those of the aqueous phase, could be seen clearly. The major portion of the radioactivity in the feces was excreted in the first 4 days, and traces continued to be eliminated for the entire experimental period.

The concentration of radioactivity in the *blood* was highest at 6 hours, remained fairly constant at a somewhat lower level between 12 and 48 hours, and then dropped very gradually (table 2). The amount of isotope still circulating in the blood after 32 days was quite appreciable, since most of a given dose was excreted in about 2 days. The isotope in *liver* also reached a peak at 6 hours and decreased progressively thereafter. This is true even though at this time not all the injected compound was absorbed and more was, therefore, coming into circulation. The percentage of the liver activity bound to the *proteins* increased progressively, from 12 percent at 6 hours to 100 percent at 4 and 8 days, and decreased thereafter to 87 and 50 percent at 16 and 32 days, respectively. The isotope concentration in the soluble and insoluble liver proteins was considerable, even at 6 hours, the earliest period of observation in this study. The specific activity increased to a broad peak centered about the 1-day period; thereafter the specific activity of the soluble proteins declined slowly to the 4th day.

The insoluble proteins showed a slightly higher rate of loss of activity. Dyer and Morris (*3*) also found maximal binding at 6- and 24-hour periods by the use of N^{15}-labeled 2-FAA. After day 4 the activity of both proteins dropped. A semilogarithmic plot of activity versus time exhibits initially a steep variable slope which levels off in the later period. Since the plot is not linear, the loss of activity must be a complex phenomenon that may involve several types of proteins, or partial progressive release of labeled metabolites from different sites of attachment to proteins.

The soluble proteins showed a higher specific radioactivity than the insoluble proteins, an observation generally made in most of our experiments. However, the activity at 6 hours was slightly higher in the insoluble protein fraction. Earlier studies on 2-FAA labeled with isotopic nitrogen gave a similar picture (*3*). The different rates of binding in the soluble and insoluble fractions suggest that the mode of binding could be dissimilar.

The question arose whether the *p*H used for the homogenization of the tissue influenced the amount of activity bound to the soluble and insoluble proteins.[8] In most of our experiments the liver was homogenized in buffer of *p*H 5 because the soluble proteins can be heat-coagulated readily at that *p*H. Since the *p*H of living matter is higher, and because other investigators have used higher *p*H values, a comparative study was performed. Aliquots of the same liver of a rat receiving injections of C^{14}-labeled 2-FAA showed a larger amount of activity in the super-

[8] We are indebted to Dr. H. A. Sober, of this laboratory, and to Dr. S. Sorof, of the Institute for Cancer Research, Philadelphia, Penna., for valuable discussions regarding this point.

natant when the homogenate was prepared at *p*H 7.8 rather than 5 (table 3). Coagulation of the supernatant gave also somewhat larger weights of protein at *p*H 7.8, *e.g.*, 243 mg versus 171 mg. Yet the specific activity of the soluble protein was about 10 percent lower at *p*H 7.8 as compared to that at *p*H 5. The specific activity of the insoluble proteins was similar in both cases. The total activity in the soluble protein was larger at *p*H 7.8 mainly because of the larger amount of protein soluble at the higher *p*H; however, the activity in the soluble proteins at *p*H 5 and 7.8 represented an equal proportion (72%) of the activity in the supernatants from which these proteins were precipitated. The total activity of the insoluble proteins was slightly less at *p*H 7.8 than at *p*H 5.

TABLE 3.—Effect of *p*H on radioactivity bound to soluble and insoluble liver proteins of a rat receiving an injection of 2-FAA*

	Total activity		Weights of proteins		Specific† and total‡ activity			
	Homogenate (cpm × 10^{-3})	Supernatant (cpm × 10^{-3})	Soluble (mg)	Insoluble (mg)	Soluble protein§ cpm/mg (mμmoles/g)	Percent	Insoluble protein cpm/mg (mμmoles/g)	Percent
*p*H 5	702	280	171	518	1190 (178)	29	711 (106)	52
*p*H 7.8	723	356	243	502	1060 (158)	36	707 (106)	49

* A female Buffalo strain rat, weight 130 g, received an intraperitoneal injection of 13 mg of C^{14}-labeled 2-FAA (3.8×10^6 cpm) and was killed after 48 hours. The liver was divided into two 3.5 g aliquots. One was homogenized in 14 ml 0.1 M acetate buffer, *p*H 5, the other in 14 ml 0.1 M phosphate buffer, *p*H 7.8, in a Potter-Elvehjem apparatus. The homogenates were centrifuged for 1 hour (Spinco Model L centrifuge, #30 head, 30,000 rpm). The pellets were resuspended in 14 ml of the respective buffers, and centrifuged. The supernatants were pooled, and the soluble proteins coagulated by being heated for 10 minutes on a steam bath. (The solution of *p*H 7.8 was adjusted to *p*H 5 with acetic acid.) The proteins were washed with ethanol, then extracted in a Soxhlet with ethanol for 48 hours.

† The specific activity is in cpm/mg and in parentheses in mμmoles/g.

‡ Total activity of proteins is expressed in percent of the activity in homogenate.

§ The soluble proteins represented 72 percent of the activity in the supernatant at *p*H 5 and 7.8.

Hydrolysis of Liver Proteins Containing Bound Radioactivity

Acid or alkaline hydrolysis of the proteins described in the experimental part resulted in the liberation of 50 percent of the radioactivity as materials extractable into the ether-ethanol phase from an aqueous phase made to 50 percent saturation with $(NH_4)_2SO_4$. A black, humin-like precipitate in the aqueous phase after acid hydrolysis accounted for 20 percent of the initial activity in the protein.

Exhaustive hydrolysis by enzymes for 6 to 12 days followed by extraction of the solution containing $(NH_4)_2SO_4$ with ether-ethanol likewise transferred 50 to 60 percent of the radioactivity to the organic phase. Alternatively, when the hydrolysate was extracted sequentially with ether, ether-ethanol, and *n*-butanol without salting out the aqueous phase, 5 to 8, 2 to 4, and 40 to 50 percent, respectively, of the total protein

radioactivity was found in the organic solvents. The amount of products extractable into solvents was the same with any of the enzyme preparations used (*see* "Methods," p. 155). Thus, the enzymic method yielded results similar to the chemical hydrolysis in terms of the total proportion of protein-bound radioactivity rendered soluble in organic solvents. It can be concluded that the enzymic method as performed was adequate to release any bound metabolite capable of being so freed.

Further confirmation of this concept was afforded by a number of complementary experiments. Dialysis against distilled water of the aqueous phase after extraction of the organic-soluble materials led to the transfer of about 20 to 30 percent of the activity to the dialysate. However, only 10 percent of the dialysate or 3 percent of the total activity was organic-soluble. Furthermore, reincubation of the nondialyzable bag contents with fresh enzyme rendered only small amounts of the isotope extractable by the series of solvents, which indicated that the first hydrolysis had already gone as far as possible. Acid hydrolysis of an aqueous phase after extraction of an enzymic hydrolysate led to a 40 percent loss of isotope.[9] The balance of the radioactivity was distributed as follows: 30 percent in a black, insoluble precipitate, 25 percent in a soluble part which in turn was subdivided into a fraction extractable into ether-ethanol, 7.8 percent, and *n*-butanol, 4.5 percent, and the balance in the aqueous phase. It would appear, therefore, that the additional acid hydrolysis gave little material which resembled the compound administered, the known urinary metabolites of 2-FAA, or even the material liberated from the proteins by the first hydrolysis.

In this connection, incubation of the enzymic hydrolysate of a protein with Taka-diastase or bacterial β-glucuronidase did not free any additional organic-soluble products. In addition, an enzymic hydrolysate was treated as described for the separation of free compounds, sulfates, and glucuronides in urine, namely chromatography on alumina of an ether-ethanol extract from a hydrolysate brought to 50 percent saturation with $(NH_4)_2SO_4$ (*16*). Thus, 27 percent of the isotope in the extract was in the ether-ethanol eluate corresponding to the unconjugated compounds, 54 percent in the 50 percent aqueous ethanol eluate normally containing sulfates, and 13 percent in the aqueous buffer eluate which generally held the glucuronides. Since these types of conjugates were absent, as noted, the hydrolysate must have been composed of at least 3 different classes of materials.

Further evidence for the multiplicity of materials bound to the proteins was derived from additional *chromatographic studies*. Thus, autoradiographs of chromatograms of the ether-ethanol extracts of acid, alkaline, and enzymic hydrolysates [half-saturated with $(NH_4)_2SO_4$] in the *butanol solvent* system exhibited 5 to 6 spots (table 4). These radioactive materials are presumably due to different chemical entities. While those

[9] In some of the many hydrolytic experiments performed, a part (10-50%) of the radioactivity in the protein was likewise not accounted for in the hydrolysate. In a few cases an undigested precipitate with four- to fivefold higher specific activity than the starting material was obtained in the enzymic hydrolyses.

with similar mobilities have been placed in the table on one line, it remains to be shown that they are identical. Thus, it could be that spot #2 from the acid hydrolysate is different chemically from spot #2 after alkaline or enzymic hydrolysis. Furthermore, the relative intensities of spots in the hydrolysates were dissimilar, which suggested that the various modes of hydrolyzing the labeled proteins yielded distinct compounds with similar mobilities. Investigation of their exact chemical structure is of considerable interest and importance. However, the chromatograms do demonstrate that a number of compounds were bound by several different liver proteins.

TABLE 4.—Paper chromatography of extracts of protein hydrolysates*

	Hydrolysate		
	Acid	Alkali	Enzyme
Spot No.	R_f value (× 100) and relative intensity †		
1	8–12 (1.3)	0–6 (1.1)	0–15 (4.8)
2	31–34 (2.0)	37–40 (6.0)	29–38 (8.9)
3	48–56 (5.5)	46–64 (12.5)	38–54 (10.6)
4	67–69 (7.8)		58–70 (48)
5	70–74 (17)	72–79 (20)	70–84 (2.0)
6	87–95 (37)	86–95 (29)	84–94 (24)

* Aliquots of the hydrolysates obtained as described in the text were applied to Schleicher and Schuell's paper No. 598 and chromatographed in ascending fashion in *sec.*-butanol-3 percent NH_4OH (*12*).

† Relative intensity of spot, expressed as percent of total activity on a strip, is given in parentheses. The radioactive spots, visualized by autoradiography on Kodak Royal Blue film, were carefully cut out and counted in a Packard Tri-Carb scintillation spectrometer. Areas of a strip without apparent radioactivity were also counted, and the total activity per strip was taken as 100 percent. The R_f value was measured from the front to the back of each spot.

Chromatography of an ether extract of an enzymic or acid hydrolysate (after acetylation) in the *cyclohexane-solvent* system resulted in spots at the origin, near the solvent front, and some slight general darkening of the strip. Furthermore, superimposed on this there were usually a few more weak-to-moderate radioactive areas, with R_f values of 0.02 to 0.06, 0.19 to 0.26, 0.36 to 0.43, and 0.53 to 0.68. The area with an R_f 0.19 to 0.26 may be similar to one noted by us as a weak spot in the chromatograms of the urinary metabolites, and observed also by Miller *et al.* in their recent comprehensive studies (*17*). If the urinary metabolite with this mobility and the one obtained from the proteins are actually identical, it would be the first instance of a derivative of protein-bound metabolite excreted in the urine. Indeed, while the main urinary metabolites of 2-FAA give rise to protein-binding (*11,18*), they cannot be detected as such in the protein hydrolysates.

The spot with an R_f value of 0.53 to 0.68 suggested the presence of 2-FAA. Another, with a mobility of 0.37 to 0.45, might have been indicative of 2-fluorenamine. This spot was noted only after enzymic hydrolysis, since any free amine would have been acetylated in the procedure used after the acid hydrolysis. The presence of both these compounds was confirmed by the accurate and specific isotope dilution method

(*13*) on an ether extract of an enzymic hydrolysate. This experiment revealed that low amounts equivalent to 0.31 and 0.72 percent of the total radioactivity in the protein were due to 2-FAA and 2-fluorenamine, respectively. It is difficult to believe that the small quantities so found were actually bound to certain proteins by a covalent bond. These compounds more likely were trapped by the protein, perhaps by surface phenomena, and therefore not extracted by the Soxhlet procedure used in demonstrating so-called protein-bound radioactivity.

Chromatography on a silicic acid column, so successful for resolving the ether-soluble urinary metabolites of 2-FAA (*12*, *14*), confirmed the results obtained by paper chromatography. Thus, after application of an ether extract of a protein hydrolysate to the column, the plot of activity versus volume of eluate in no way resembled the elution curve noted with the urinary metabolites. With a 60 g silicic acid column 30 cm high, most of the activity appeared as a single peak with a long tail, centered about an eluate volume of 50 ml. A second low but broad peak was eluted around 260 ml. The final ethanol eluate gave the same spots on a paper chromatogram (butanol solvent system) as the ether extract (table 4). Rechromatography of the first two peaks on a 60-cm-high column (120 g of silicic acid) again resulted in the elution of the major part of the activity applied in the early fractions as four broad peaks centered around 80, 160, 260, and 620 ml. Paper chromatograms of all those fractions in the cyclohexane system showed activity near the solvent front. In addition, the first fraction was characterized by a long tail reaching almost back to the origin; it contained a material which accelerated the movement of solvent along this band. Fractions 2 and 3 revealed material with the mobility of 2-FAA and 2-fluorenamine, though inspection of the autoradiograph suggested that the spot near the solvent front accounted for a larger part of the activity in this fraction. Fraction 4 exhibited a darker area with a mobility of 0.28 to 0.38, corresponding perhaps to the spot with an R_f value of 0.19 to 0.26 described earlier; slight variations in mobilities on different chromatograms were encountered in previous studies (*12*).

Artifact Binding

Homogenization, in a Waring blendor or in a Potter-Elvehjem type apparatus, of liver with C^{14}-labeled 2-FAA or with the hydroxylated derivatives gave rise to very small amounts of radioactivity, from traces to at most 40 mμmoles of compound per g of dry liver protein bound to the soluble, insoluble, or boiled fractions of liver proteins (table 5). The type of indiscrimate and low binding may be due to an adsorption of traces of labeled materials to the surface of the protein either during homogenization or precipitation.

An entirely different picture prevailed with the aminofluorenols. 2-Fluorenamine gave rise to a small amount of bound isotope where homogenization was performed in the Potter-Elvehjem apparatus, and

TABLE 5.—Amount of radioactivity bound by liver proteins during homogenization under various conditions

Compound†	Specific activity cpm/ mμmole	Radioactivity, expressed as mμmoles of compound bound per g of dry liver protein fraction*					
		Waring blendor			Potter-Elvehjem homogenizer		
		Soluble	Insoluble	Boiled	Soluble	Insoluble	Boiled
2-FAA	375	10	20	20	10	20	10
1-OH-2-FAA	43	T	T	T	T	T	T
3-OH-2-FAA	252	T	20	30	10	T	10
5-OH-2-FAA	543	40	30	20	20	20	30
7-OH-2-FAA	435	20	30	20	20	20	30
8-OH-2-FAA	65	T	T	T	T	T	T
2-Fluorenamine	355	60	70	60	40	40	50
2-Amino-1-fluorenol	43	690	1220	560	350	250	290
2-Amino-3-fluorenol	168	1570	770	360	400	510	310
7-Amino-2-fluorenol	435	930	380	360	230	310	180

*T = Trace. Small amounts thus designated were not the result of insufficient mixing of the homogenate and the compound. Compounds were added to each tube as powders or as solutions (*see* "Methods"). To keep the table reasonably simple, data on the total activity in each group are not given. However, the radioactivity recovered in the aqueous and ethanol washes of the proteins accounted for the amount of isotope contained in the 1.5 or 1.0 mg of compound, respectively, used in each experiment. It can be concluded that the labeled compound was properly incorporated into the homogenate.

†Abbreviations: *N*-(1-hydroxy-2-fluorenyl)acetamide = 1-OH-2-FAA, etc.

a somewhat higher level with the blendor in the 3 types of proteins used. However, considerable amounts of radioactivity were bound with 2-amino-1,-3-, and -7-fluorenol. Of these, the 3-hydroxy derivative contributed the largest amount of bound activity over-all, the 1-hydroxy compound a slightly lower level, and the 7-hydroxy derivative even somewhat less. With these compounds appreciable differences were noted between proteins prepared by homogenization in the Potter-Elvehjem or the Waring blendor. The latter mode of sample preparation gave in all cases the proteins with the higher activity. Perhaps the increased binding of radioactivity from the easily oxidized aminophenols can be attributed, in part, to air entrainment by the suspension in the vortex of the high-speed blendor. Such oxidation would be facilitated by the large, newly forming surfaces during the rapid and thorough mincing of the tissues and could lead to insoluble polymeric substances (*10*) or to reactive oxidized intermediates which could spontaneously react with cell constituents (*19*, *20*). Such phenomena would be much less pronounced in the Potter-Elvehjem homogenizer, where the rapidly rotating pestle below the liquid surface does not cause the aeration of the suspension typical of the Waring blendor. It may be observed that 2-amino-1-fluorenol produced a higher level of activity bound to the insoluble proteins in the blendor, whereas the other substances showed a higher activity in the soluble proteins. It could be that the more readily oxidized 1-hydroxy derivative yielded a larger proportion of insoluble polymeric materials, while the other compounds resulted in the production of lower molecular weight compounds which can be solubilized by the protein solution. The fact that the Waring blendor was made of metal and the Potter-Elvehjem homogenizer consisted of glass

and teflon may also influence the amounts of radioactivity bound to protein. An effect of metal equipment on the binding of carcinogen derivatives has been observed in another laboratory.[10]

It should be emphasized that while the binding occurred to fresh tissue, precautions were taken to keep the homogenizers and solutions cold during the mincing and subsequent treatment. Thus it must be assumed that true enzymic reactions were minimal, and whatever binding took place was not the result of incorporation by such mechanisms. Further confirmation of this thesis can be found in the fact that binding was demonstrated with boiled liver. The highest binding with the heat-denatured protein was found with 2-amino-1-fluorenol in the Waring blendor, the lowest with the 7-hydroxy compound in the Potter-Elvehjem homogenizer (table 5).

A further experiment was performed to investigate the possible *role of the homogenization* in the artifact-binding. If the grinding and air entrainment associated with the homogenization were essential, then no binding should be observed when the soluble proteins and the radioactive aminofluorenol simply were mixed. The test showed, however, that considerable binding occurred in the presence or absence of atmospheric oxygen and at various time intervals (table 6). The binding was instantaneous and no increase with time took place. The solutions were kept in an ice bath at all times, which minimized any true enzymic reaction. Only about 1 percent of the radioactivity was bound; the remainder was recovered in the supernatant after precipitation of the protein by heat. Thus, it would seem that this reaction follows quite definite stoichometric rules. In any case, the binding of isotope to the proteins by these techniques must be considered as an artifact and is of a different type from that observed *in vivo*. Preliminary investigations on the hydrolysis of such proteins failed to render any of the isotope extractable into an organic solvent, whereas parallel experiments with radioactivity bound *in vivo* rendered about 50 percent so extractable (*see* p. 162).

TABLE 6.—Protein-bound radioactivity obtained by mixing C^{14}-labeled 7-amino-2-fluorenol and soluble proteins*

Time (hours)	Atmosphere	Protein		Supernatant total activity cpm$\times 10^{-4}$
		Specific activity mμmoles/g	Total activity cpm$\times 10^{-4}$	
0	Air	1, 600	3. 8	209
0	Nitrogen	1, 200	2. 7	247
1	Air	860	2. 1	193
1	Nitrogen	880	2. 2	218
24	Air	920	2. 0	211
24	Nitrogen	860	1. 9	206

*The supernatant of a homogenate, prepared in a Potter-Elvehjem apparatus, of 11.4 g of liver was mixed with the labeled aminofluorenol. Each tube contained 1.5 mg of compound (215×10^4 cpm) and protein solution from 1.4 g of liver.

[10] Personal communication from Drs. C. Heidelberger and A. R. Somerville, University of Wisconsin, Madison, Wis.

DISCUSSION

One aspect of these studies dealt with the effect of dose and time on the fate of 2-FAA. From the fact that the number and amounts of the urinary metabolites were similar, within the limitations of the experimental techniques used, despite a 1000-fold range of dosage administered, it can be concluded that the biochemical reactions such as hydroxylation, conjugation, deacetylation, and interactions with tissues occur either practically simultaneously or sequentially, but without competitive pathways. If these pathways played a role, a dose-dependent channeling along a favored route should have been noted, *i.e.*, as the level of compound administered was reduced, the amounts of some metabolites should have decreased more than that of others. This was not true. Likewise, radioactivity firmly attached to the liver proteins was found at all dosage levels which suggested that the reactions resulting in this combination were also noncompetitive. The significance of the rather constant proportion of the total liver activity, about two thirds, bound to the liver proteins despite the decreasing level of the dose administered, is presently obscure. It does suggest, however, that at the 1-day period this proportion of the dose has been converted to metabolites able to bind, or alternatively, that during this 1-day period a number of binding sites equivalent to the amount observed has been generated. Again, both of these possibilities may be operative.

The data in table 2, showing an increase in the proportion of the liver activity bound to proteins as a function of time, support these concepts. Thus, at the early periods, the limiting factor may be the amounts of the metabolites capable of binding, whereas the slow increase to 100 percent of the activity firmly bound between days 1 and 4 may reflect creation of binding sites. It will be recalled that the composition of the urinary metabolites appeared to be invariant with time, but it is possible that the metabolites involved in the protein-binding are not those found in the urine. In any case, the data obtained in these studies would suggest that the binding of radioactivity from the metabolites of 2-FAA occurs both to preformed proteins and during synthesis of new proteins.

The data of Dyer and Morris (*3*) and those reported by Seal, Irving, and Gutmann (*21*) at the meeting of the American Association for Cancer Research in 1959, on the binding of radioactivity from 2-FAA versus time, generally support those presented herein. Furthermore, Hadler and Lee (*22*) observed that the proportion of radioactivity from 7,12-dimethylbenz[*a*]anthracene bound to the liver proteins increased to a maximum at 1 day and then decreased slowly. Heidelberger and Moldenhauer (*23*) noted a maximum of activity bound to the skin proteins of mice at 1 to 2 days with a variety of hydrocarbons. Curves representing the decline in protein-bound activity were, however, not linear. The studies with the carcinogenic azo dyes likewise involved a maximum of binding with time, but the decrease of protein-bound azo dye in livers of rats followed a linear pattern with a half life of about 4 days (*24*, *25*). The azo dye-protein combination may be a simpler system than the one

with activity from 2-FAA and liver proteins, for in our experiments the decrease of activity in the liver proteins was nonlinear. Other contemporary investigations have, likewise, not yet provided a clear-cut answer regarding the mode of binding of carcinogens to tissue constituents (*7–9, 17, 26*). Further studies to sort out these complex reactions, especially with a view to determine which one is involved in carcinogenesis, is an important future goal.

The use of 2-FAA of high specific radioactivity has permitted detection of significant amounts of activity 32 days after a single dose of the labeled compound. An interesting finding was the relatively small variation of the activity in the blood, from 38 to 5.4 mμmoles/ml at 6 hours and 32 days, whereas the activity in the liver and in the urine was very high at the early periods and very low later. It would appear that the blood has a limited total capacity for the metabolites of 2-FAA, so that in the early stages the tissues form the main reservoir of these compounds. As excretion lowers the amounts of the metabolites, the affinity of certain components of the blood for them assumes relatively more importance so that the blood level of the activity decreases very slowly. This affinity must be fairly high, for otherwise the kidneys would be able to transfer the metabolites to the urine. The nature of the metabolites in the blood and the type of carrier remain to be established. Avigan (*27*) has demonstrated an association of certain carcinogenic hydrocarbons and of 2-FAA and related compounds with serum lipoproteins.

In view of the demonstration that certain metabolites of 2-FAA, namely the aminofluorenols, lead to protein-bound radioactivity as an artifact, the problem arises as to how much of the total radioactivity bound to proteins observed during experiments *in vivo* is due to this type of binding. While the aminofluorenols account for only a small proportion of the urinary metabolites, it could be that larger amounts are produced but that a portion is trapped by tissue constituents at the site of formation. It seems likely, however, that a large part of the protein-bound activity can be ascribed to reactions involving other derivatives of 2-FAA. Hadler *et al.* (*28*) have described and discussed cases involving the binding of materials derived from certain carcinogens to tissue constituents, possibly as a result of an artifact-type of reaction.[11] Further refinements in technique, the object of research in a number of laboratories, may lead to better discrimination between real and artifactitious binding, and also between binding due to general metabolic reactions and those specifically involved in the carcinogenic process.

The results obtained in this study regarding the nature of the metabolites of 2-FAA bound to the tissue constituents form an interesting parallel to those found with the carcinogenic azo dyes (*24*). With the dyes, the compounds isolated and identified after the hydrolysis of the protein complexes were 4-aminoazobenzene (*p*-phenylazoaniline) and the monomethyl de-

[11] Another example of artifact-binding involving mouse epidermis and anthanthrene was recently reported (Daudel, P., Nectoux, F., Pichat, L., and Prodi, G.: Fixation sur les protéines cellulaires de l'anthanthrène marqué par du radiocarbure. Comp. rend. Acad. sc. 251: 1049–1051, 1960).

rivative thereof. Both of these are known metabolites of the administered 4-dimethylaminoazobenzene (*N,N*-dimethyl-*p*-phenylazoaniline). In addition, the protein hydrolysate contained so far unidentified polar dyes (*29*). Data recently reported by Hughes (*30*) suggest that the polar dye might be substituted by tryptophan, valine, and perhaps cysteine. With 2-FAA, small amounts of 2-fluorenamine and 2-FAA itself were positively identified in the hydrolysates of the proteins. The bulk of the radioactivity consisted, however, of a number of more polar compounds, the chemical nature of which remains to be elucidated. Likewise, similar studies with the carcinogenic hydrocarbons have yet to yield significant results with respect to the nature of the complexes involved in carcinogensis despite considerable efforts exerted on this crucial point (*31*).

While the studies reported and discussed herein have emphasized the binding of isotope from the carcinogen to protein, combination with other tissue constituents may be equally likely and important in the elicitation of the carcinogenic process. For example, Dr. E. Farber reported at the meeting of the American Association for Cancer Research, April, 1961, that activity from a number of carcinogens, including 2-FAA, was bound to liver ribonucleic acid (*32*). Future investigations along all these lines will be of considerable interest.

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Effect of Hormones on Mammary-Tumor Development From Transplanted Hyperplastic Alveolar Nodules in Hypophysectomized-Ovariectomized-Adrenalectomized C3H/Crgl Mice[1, 2]

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SUMMARY

Hyperplastic alveolar nodules were transplanted into the mammary-gland-free fat pads of young C3H/Crgl female mice. The host animals were then hypophysectomized-ovariectomized-adrenalectomized and treated with various combinations of estradiol-17β, corticoids, and somatotropin. A combination of deoxycorticosterone + corticosterone + somatotropin permitted the development of tumors from the transplanted nodules. Both adrenal steroids were necessary, with somatotropin, to permit tumorigenesis. Large amounts of cortisol resulted in lactogenesis in the nodules and inhibited neoplastic development. Estrogen, while not necessary for tumor formation, enhanced the tumorigenic action of corticoids + somatotropin.—J. Nat Cancer Inst. 27: 173–185, 1961.

SINCE THE development of a method for the transplantation of mammary tissues into gland-free fat pads (*1*), a variety of experiments has been carried out in our laboratory to determine the characteristics of precancerous mammary nodules in C3H/Crgl mice. When transplanted into intact females, nodules, but not normal mammary tissues, give rise to hyperactive outgrowths and frequently develop into tumors within a short time (*1*, *2*). We recently delineated various combinations of hormones that can support tumor development from transplanted hyperplastic nodules in hypophysectomized-ovariectomized mice (*2*). The results from these experiments showed that the neoplastic transformation of transplanted nodules 1) possibly did not require estrogen, 2) was inhibited by cortisol, and 3) required either somatotropin (STH) or mammotropin (MH), as well as a mineralocorticoid. It is known that in virgin female mice little, if any, MH is secreted (*3*, *4*). Since tumors develop in virgin females of the C3H strain, we concluded that STH was the most important

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pituitary hormone directly involved in tumorigenesis in virgin animals. For this reason, we have used STH as the only pituitary hormone in the experiments described in this paper.

The interpretation of our previous results was complicated by the fact that the adrenal glands were present in the experimental animals. Therefore, to elucidate further the effects of estrogen, adrenocortical steroids, and STH on the growth and neoplastic development of transplanted nodules, the present series of experiments has been conducted with hypophysectomized-ovariectomized-adrenalectomized mice.

MATERIALS AND METHODS

The animals used were female mice of the C3H/Crgl strain, which is characterized by a high incidence of mammary cancer in both virgin and breeding females.

Individual hyperplastic alveolar nodules in which lactation had been induced by exogenous hormone treatment were selected from multiparous, tumor-bearing, nonpregnant C3H mice and transplanted into both gland-free #4 fat pads of 3-week-old female mice. Each donor mouse provided nodules for several recipients. The techniques have been described previously (*1*, *2*). To allow vascularization of the transplants, the host animals were left undisturbed for 2 weeks, when they were hypophysectomized-ovariectomized-adrenalectomized. We removed the ovaries and adrenals through a single mid-dorsal incision 1 day prior to hypophysectomy and began hormone treatments 1 day after hypophysectomy. The animals were killed 12 weeks after initiation of treatment. The operative techniques and postoperative care are described elsewhere (*5*).

The hormones used in these experiments were estradiol-17β (E), corticosterone acetate (B), cortisol acetate (F), deoxycorticosterone acetate (DCA), and STH. Bovine STH was generously supplied by Professor C. H. Li (*6*). This STH preparation is reported to contain 0.5 to 1 percent MH and 1 percent other pituitary hormones.

The hormones were administered in daily subcutaneous injections of 0.2 ml aqueous suspension (steroids) or solution (protein). When more than 1 steroid was administered, the hormones were combined in a single injection of 0.2 ml. The daily doses of hormones were as follows: E, 1 μg (days 1–64), 2 μg (days 65–83); B, 125 μg; F (low) 10 μg; F (high), 125 μg; DCA (low), 125 μg; DCA (high), 500 μg; STH, 250 μg (days 1–10), 400 μg (days 11–20), 500 μg (days 21–45), and 750 μg (days 46–83).

The experimental animals were separated into the following groups:

(A) Mice treated with corticoid(s) + STH
Group 1—11 mice treated with DCA (high) + STH
Group 2—5 mice treated with DCA (high) + B + STH
Group 3—5 mice treated with DCA (low) + B + STH
Group 4—3 mice treated with DCA (low) + F (high) + STH

(B) Mice treated with E + corticoid(s) + STH
Group 5—6 mice treated with E + DCA (high) + STH
Group 6—5 mice treated with E + B + STH
Group 7—4 mice treated with E + B + DCA (low) + STH
Group 8—4 mice treated with E + F (high) + DCA (low) + STH
Group 9—6 mice treated with E + F (low) + DCA (low) + STH

The number reported in each group includes only those animals in which the operations were successful and which survived the full experimental period. At necropsy, we examined each animal carefully under a dissecting microscope to insure the completeness of the surgical procedures. The excised skin with mammary glands attached was fixed overnight in 15 percent formalin, after which the mammary glands were removed, defatted, stained with hematoxylin, dehydrated, and cleared. Under a dissecting microscope, the associated connective tissue and muscle were removed. The glands were then stored in methyl salicylate prior to further examination and photography. We noted the extent of ductal and alveolar growth in the host glands, as well as the presence or absence of hyperplastic alveolar nodules, and recorded the growth and neoplastic development of each transplanted nodule.

In general, the tumor incidence was so low that it was not feasible to make statistical analyses of the differences among individual groups. However, we used the Fisher exact-probability test wherever possible, and the results of the analysis are given in the Discussion.

RESULTS

Owing to the difficulty of keeping untreated hypophysectomized-ovariectomized-adrenalectomized mice alive for 12 weeks, no untreated controls were used in the present experiments. Previous work showed that no nodule transplants gave rise to tumors in untreated hypophysectomized or hypophysectomized-ovariectomized females (*2*); accordingly, we have assumed that transplanted nodules would not develop into tumors in hypophysectomized-ovariectomized-adrenalectomized controls. The results of hormonal treatments are summarized in the following paragraphs and in the tables.

(A) Mice Treated With Corticoid(s) + STH (Table 1)

Group 1.—Eleven hypophysectomized-ovariectomized-adrenalectomized mice were treated with DCA + STH for 12 weeks. Of the 22 transplanted nodules, 3 regressed and the rest were maintained (fig. 1). Some of these had outgrowths of the ductal or lobuloalveolar type. One nodule was filled with milk.

Ductal branching in the host glands was like that found in 5- to 6-week-old females.

Groups 2 and 3.—Five hypophysectomized-ovariectomized-adrenalectomized mice were treated with DCA (500 μg/day) + B + STH (Group

2) and 5 with DCA (125 μg/day) + B + STH (Group 3). In Group 2 all nodules were maintained and many appeared like small, expanding nodules (*1*). The latter possessed dilated alveoli and 2 were filled with milk. Three of the nodules had small hyperactive outgrowths (fig. 2), and 2 gave rise to tumors.

In Group 3, which was given the lower dose of DCA, 4 of the 10 nodule transplants regressed. Five were maintained, 2 of which were filled with milk. One nodule gave rise to a tumor (fig. 3).

The ductal branching in the host glands of Groups 2 and 3 was similar to that in Group 1.

Group 4.—Three hypophysectomized-ovariectomized-adrenalectomized mice were treated for 12 weeks with DCA (low) + F (high) + STH. Of the 6 transplanted nodules, 3 regressed completely. The other 3 grew slightly and showed dilated alveoli filled with milk. No tumors were observed.

The host glands were similar to those of Groups 2 and 3.

TABLE 1.—Effect of corticoid(s) + STH on nodules transplanted into mammary-gland-free #4 fat pads of C3H/Crgl female mice. Hosts hypophysectomized-ovariectomized-adrenalectomized and treated with hormones, beginning at age of 5 weeks, terminating at age of 17 weeks

		Nodule transplants			
Group	Daily hormonal treatment*	Total No.	Number of surviving transplants	Number producing tumors	Remarks
1	DCA (high) + STH	22	19	0	Nodules maintained; few showed small outgrowths; 1 was filled with milk; host gland immature
2	DCA (high) + B + STH	10	10	2	Many behaved like expanding milk-filled nodules; 3 had small outgrowths; host glands like Group 1
3	DCA (low) + B + STH	10	6	1	Five nodules maintained (2 were milk-filled); host glands like Group 1
4	DCA (low) + F (high) + STH	6	3	0	Nodules grew slightly and were filled with milk; host glands like Group 1

*See text for doses of hormones used.

(B) Mice Treated With E + Corticoid(s) + STH (Table 2)

Group 5.—Six hypophysectomized-ovariectomized-adrenalectomized mice were treated for 12 weeks with E + DCA (high) + STH. Most of the nodule transplants gave rise to small lobuloalveolar outgrowths, and 2 of these outgrowths developed palpable tumors. However, the outgrowths were smaller than those observed in similarly treated hypophysectomized-ovariectomized females (*2*).

TABLE 2.—Effect of E + corticoid (s) + STH on nodules transplanted into mammary-gland-free #4 fat pads of C3H/Crgl mice. Hosts hypophysectomized-ovariectomized-adrenalectomized and treated with hormones, beginning at age of 5 weeks, terminating at age of 17 weeks

Group	Daily hormonal treatment*	Nodule transplants: Total No.	Number of surviving transplants	Number producing tumors	Remarks
5	E + DCA (high) + STH	12	12	2	Lobuloalveolar outgrowth from nodules; host glands with small lobules and 18 nodules
6	E + B + STH	10	10	2	Nodules maintained; few with small outgrowths; host glands small but with some alveoli and lobules
7	E + B + DCA (low) + STH	8	8	4	As in Group 6
8	E + F (high) + DCA (low) + STH	8	6	0	Nodules grew like milk-filled expanding nodules; host glands as in Group 6
9	E + F (low) + DCA (low) + STH	12	11	1	Most nodules maintained with slight outgrowth from 3 nodules; host glands with good ductal and alveolar development; 1 animal with 3 nodules

*See text for doses of hormones used.

The host glands, consisting of many small lobules, filled the whole fat pad. Eighteen hyperplastic alveolar nodules were counted among the host glands (fig. 4) in 4 of the 6 animals.

Group 6.—Five hypophysectomized-ovariectomized-adrenalectomized mice were treated for 12 weeks with E + B + STH. Four transplanted nodules did not grow, 2 grew as small expanding nodules, 2 showed small hyperactive outgrowths (fig. 5), and 2 gave rise to tumors. The alveoli of all nodules were dilated and filled with watery secretion.

The host glands were small, but some alveoli and lobules were present at the ends of the ducts.

Group 7.—Four hypophysectomized-ovariectomized-adrenalectomized mice were treated with E + B + DCA (low) + STH for 12 weeks. Of the 8 nodule transplants, 2 were maintained and 2 showed small lobuloalveolar outgrowths. The remaining 4 nodules gave rise to 1 large and 3 small tumors (fig. 6).

The host glands were small, occupying only part of the fat pad, but a few small, aberrant lobules and secretory nodules (fig. 7) were present, which resembled the "noduloids" described previously (7).

Group 8.—Four hypophysectomized-ovariectomized-adrenalectomized mice were treated for 12 weeks with E + DCA + F (high) + STH. Of the 8 nodules transplanted, 2 regressed. The other 6 grew slightly as expanding nodules and became filled with a milklike secretion (fig. 8). One of these gave rise to a small ductal outgrowth. No tumors were observed.

593953—61——21

The host glands were small, but contained a few "noduloid" structures and many aberrant lobules filled with serous secretion.

Group 9.—Six hypophysectomized-ovariectomized-adrenalectomized mice were treated with E + DCA + F (low) + STH for 12 weeks. One of the transplanted nodules regressed, 8 were maintained, and 3 gave rise to small lobuloalveolar outgrowths. One nodule developed into a small tumor.

The host glands, which occupied most of the fat pad, showed greater ductal and alveolar growth than those of any other group except Group 5. The lobules, however, contained less secretion than those of the mice in Group 8. Three hyperplastic alveolar nodules were found in the glands of 1 of the host animals.

DISCUSSION

In our previous work (*2*, *8*) on the development of tumors from transplanted hyperplastic alveolar nodules in hypophysectomized-ovariectomized mice, we found that exogenous estrogen was unnecessary for the neoplastic transformation, which could take place in animals treated only with DCA + STH. However, our present experiments show that when this hormone combination was administered to hypophysectomized-ovariectomized-adrenalectomized mice (Group 1), no tumors arose from the transplanted nodules, and the nodule outgrowths were much smaller than those in similarly treated hypophysectomized-ovariectomized animals. The frequency of the tumorous transformation in the latter group is significantly different from that in Group 1 at the 2 percent level of confidence. Evidently some factor from the adrenal supported the tumorigenic action of DCA + STH in these mice. This factor might be an estrogen secreted by the adrenal, though the lack of stimulation of uteri and vaginas in the hypophysectomized-ovariectomized animals indicated the absence of significant quantities of endogenous estrogen. Alternatively, glucocorticoids might enhance the effect of DCA + STH; however, little secretion of glucocorticoid is expected in hypophysectomized animals.

Cortisol (F) was found to inhibit tumorigenesis of nodules transplanted into hypophysectomized-ovariectomized animals (*2*). Inasmuch as the mouse adrenal gland does not secrete cortisol, but corticosterone (B) (*9*, *10*), we treated hypophysectomized-ovariectomized-adrenalectomized females with B + DCA + STH (Groups 2 and 3) to see whether B might permit the development of tumors from transplanted nodules. Two doses of DCA were used: 500 μg/day (Group 2) and 125 μg/day (Group 3). Two of the 10 transplanted nodules gave rise to tumors in Group 2, and 1 of 10 in Group 3. Although this difference is not significant, the maintenance and growth of the nodules were generally better

in animals receiving the higher dose of DCA. These results indicate that B does permit the neoplastic transformation in the presence of DCA + STH. Since these animals were deprived of all steroidogenic organs and no exogenous estrogen was administered, it can be concluded that nodule transplants can give rise to tumors in the complete absence of estrogenic steroids.

Since B and F are both glucocorticoids, it was interesting to compare further their effects on tumorigenesis, which appear to be different. Hypophysectomized-ovariectomized-adrenalectomized animals were treated with B + DCA + STH (Group 3) and F + DCA + STH (Group 4). The daily doses of B and F were the same. Because of the small number of animals in these groups, only a tentative conclusion is possible. However, we observed that in animals treated with the combination containing F (Group 4), the nodules either regressed or became filled with milk, but no tumorigenesis occurred. However, in Group 3 one tumor appeared, most of the nodules were maintained, and lactogenesis occurred in only 2 nodules.

The lactogenic effect of F in mice is known (*11*, *12*). In recent work, we found that B is much less potent than F in the induction of milk secretion. Our results here support our previous conclusion that a combination which causes lactogenesis in transplanted nodules is not capable of supporting their neoplastic transformation. It appears that B, less effective than F in lactogenesis, is more capable of supporting tumorigenesis when administered with other steroid and protein hormones.

Although corticoids and STH are sufficient to allow the neoplastic transformation even in hypophysectomized-ovariectomized-adrenalectomized mice, it was of interest to see whether estrogens would enhance the tumorigenic potential of these hormones. In Groups 5 to 9, estrogen was added to various combinations of corticoids + STH.

The addition of estrogen to the combination DCA + STH (Group 5) resulted in the formation of 2 tumors from 12 transplanted nodules, though DCA + STH alone had been completely ineffective (*cf.* Group 1). E + DCA + STH also stimulated the development of hyperplastic alveolar nodules in the host glands to an even greater extent than in similarly treated hypophysectomized-ovariectomized females. No explanation for this effect can be given, especially in view of the fact that the outgrowths from transplanted nodules were larger in the hypophysectomized-ovariectomized animals.

The administration of E + B + STH (Group 6) resulted in tumor formation equivalent to that found with E + DCA + STH, even though the dose of B was less than the dose of DCA used in Group 5. However, in Group 6 there was no increase in ductal growth in the host glands, and only a few alveoli and lobules were present. In contrast, the E + DCA + STH-treated animals showed extensive ductal and alveolar development in the host mammary glands.

As one might expect, the administration of E + B + DCA + STH (Group 7) resulted in the greatest number of tumors (4 of the 8 nodules

developed into tumors). When B was replaced by an equivalent amount of F, however, lactogenesis occurred in the nodules and no tumors were formed. Decreasing the quantity of F in the combination E + F + DCA + STH (Group 9) permitted the formation of 1 tumor from 12 nodule transplants and prevented milk secretion in the remainder. The tumor incidence in this group was not significantly different from that of animals treated with E + DCA + STH alone, and the response of the host glands was similar.

The tumor incidence observed was too low to reveal a significant difference in individual groups treated with estrogen compared with similar groups without estrogen. However, if Groups 1 and 3 are combined and compared with Groups 5 and 7 combined, the frequency of the tumorous transformation is significantly higher in the estrogen-treated groups at the 2 percent level of confidence.

We have demonstrated that estrogen is not required for the neoplastic transformation of transplanted hyperplastic alveolar nodules in C3H/Crgl mice, though the presence of both DCA + B and STH is necessary. However, estrogen appears to enhance significantly the tumorigenic effect of corticoids + STH. As indicated in our previous experiments, doses of F which result in lactogenesis of the transplanted nodules inhibit their neoplastic transformation.

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PLATES

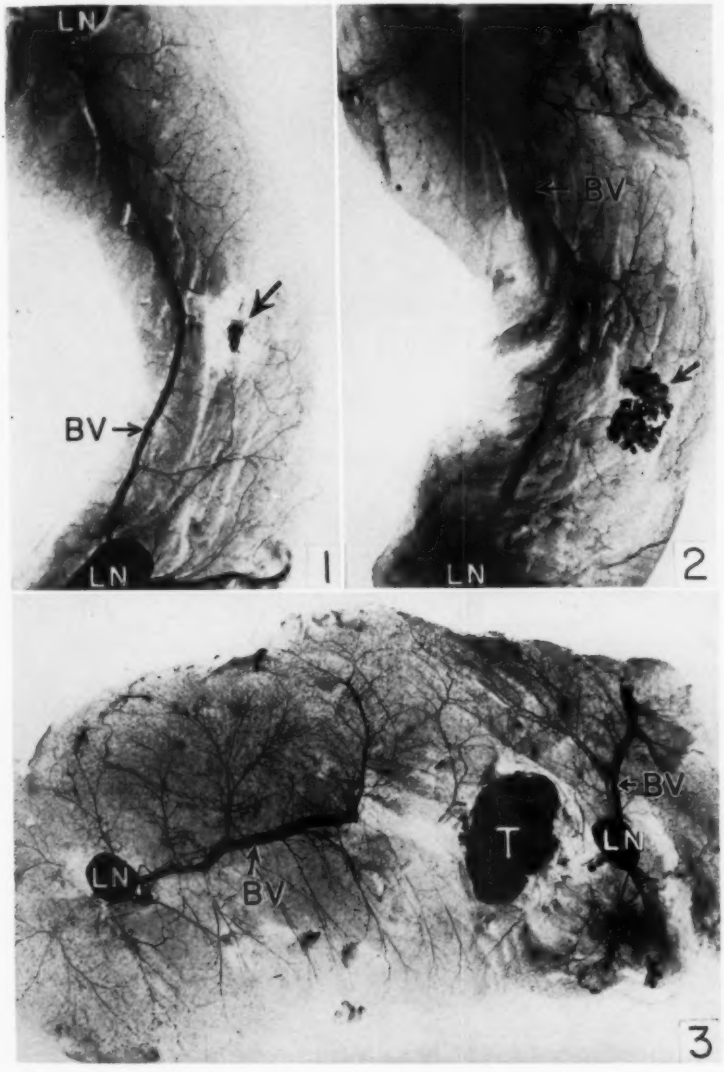
Figures in plates show wholemount preparations of mammary-gland-free #4 fat pads containing nodule transplants and undisturbed #3 thoracic mammary glands (host glands) of C3H/Crgl female mice treated with various hormonal combinations. All these mice were hypophysectomized-ovariectomized-adrenalectomized and treated with hormones beginning at age of 5 weeks and terminating at age of 17 weeks. Hematoxylin. × 6.

PLATE 23

FIGURE 1.—Transplanted nodule after daily treatment with DCA (500 μg) + STH (250–750 μg). *Note* that nodule (*arrow*) is maintained without any outgrowth.

FIGURE 2.—Outgrowth (*arrow*) from transplanted nodule after daily treatment with DCA (500 μg) + B (125 μg) + STH (250–750 μg).

FIGURE 3.—Tumor (T) arising from transplanted nodule after daily treatment with DCA (125 μg) + B (125 μg) + STH (250–750 μg).



593953—61——22

PLATE 24

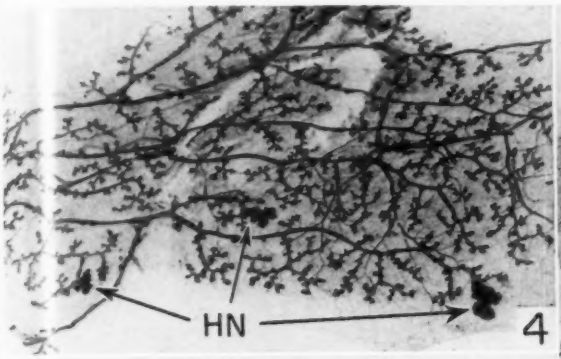
FIGURE 4.—Host gland after daily treatment with E (1 μg) + DCA (500 μg) + STH (250–750 μg). *Note* presence of hyperplastic alveolar nodules (HN).

FIGURE 5.—Outgrowth from transplanted nodule after daily treatment with E (1 μg) + B (125 μg) + STH (250–750 μg).

FIGURE 6.—Outgrowth from transplanted nodule after daily treatment with E (1 μg) + B (125 μg) + DCA (125 μg) + STH (250–750 μg). *Note* small tumors (T) present in outgrowth.

FIGURE 7.—Host gland from mouse treated as shown in figure 6. *Note* presence of small lobules or "noduloid" at *left* and large inflated nodule-like structure (*arrow*).

FIGURE 8.—Outgrowth (*arrow*) from transplanted nodule after daily treatment with E (1 μg) + F (125 μg) + DCA (125 μg) + STH (250–750 μg). *Note* enlarged milk-filled alveoli and lack of ductal elements.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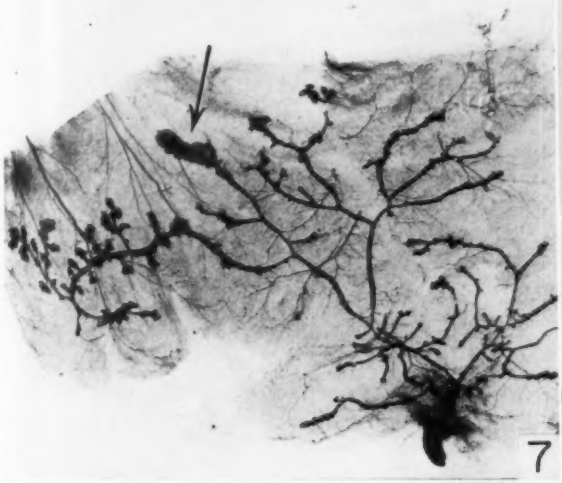
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Effect of Hormones on Maintenance of Hyperplastic Alveolar Nodules in Mammary Glands of Various Strains of Mice [1,2]

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SUMMARY

Uniparous or multiparous mice of strains C3H/Crgl, C3H/Crgl/2, RIII/Dm-Crgl, DBA/2NCrgl, A/Crgl, and A/Crgl/3, which differ in their susceptibility to mammary cancer, were hypophysectomized-ovariectomized-adrenalectomized and treated with adrenocortical or adrenocortical + adenohypophysial hormones for 15 days. Examination of untreated intact mice showed that hyperplastic alveolar nodules were present in the mammary glands of all strains. Most nodules regressed, if the hypophysectomized-ovariectomized-adrenalectomized mice were treated with deoxycorticosterone acetate (DCA) + corticosterone acetate (B) only. However, treatments with DCA + B + mammotropin and DCA + B + somatotropin were found to be equally effective in maintaining the hyperplastic nodules at a level of development similar to that of intact mice of comparable strains, and initiated lactation in some of them. There were no significant strain differences in the response of hyperplastic alveolar nodules to these two hormone combinations. It is suggested that, although the hormone requirements for nodule formation may differ in different strains, the requirements for maintenance appear to be the same, regardless of strain.—J. Nat. Cancer Inst. 27: 187-201, 1961.

IN THE C3H/Crgl strain of mice, we have found that somatotropin (STH) could replace mammotropin (MH) as a lobuloalveolar mammogen, as well as in all other phases of mammary development (*1–3*). In addition, hormone combinations that induce lobuloalveolar development in this strain result in the formation of hyperplastic alveolar nodules in the mammary glands (*2–4*). Among various strains of mice studied, STH-containing combinations have been found to act as a lobuloalveolar mammogen and lactogen only in those strains, such as C3H, RIII, and DBA, with a high incidence of mammary tumors in both virgin and parous females

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593953—61——23

(*5*). From our extrapolations of data on C3H mice, it appears likely that STH + appropriate steroids also can induce the development of hyperplastic alveolar nodules in these strains. In mice of the A/Crgl strain, however, spontaneous mammary tumors are restricted almost exclusively to breeding females, and STH-containing combinations have been found ineffective in combined lobuloalveolar mammogenesis and lactogenesis (*5*). Presumably hyperplastic nodules do not form in the mammary glands of this strain except under the influence of MH that is secreted in significant quantities only during pseudopregnancy and pregnancy (*6*).

There is considerable evidence to show that, once hyperplastic alveolar nodules are formed, both their growth potential and their hormone sensitivity differ from those of normal lobules (*3*, *7–9*). In C3H mice we have found that adrenal steroids + STH are sufficient not only to maintain such nodules, but also to permit their neoplastic transformation (*3*, *10*). In view of the altered sensitivity of nodules, it was of interest to determine whether the strain differences in the response of normal alveoli to STH are retained by the alveoli comprising hyperplastic nodules.

In the experiments reported here, we have compared the ability of MH and STH to maintain hyperplastic alveolar nodules in mouse strains which vary in their susceptibility to mammary-tumor formation. We have attempted to relate our findings to mammary tumorigenesis in these several strains.

MATERIALS AND METHODS

We used female mice of the following mammary-tumor-susceptible strains, raised at the Cancer Research Genetics Laboratory: C3H/Crgl, C3H/Crgl/2, DBA/2NCrgl, RIII/DmCrgl, A/Crgl, and A/Crgl/3. All the animals, separated from males for at least 2 weeks prior to the start of the experiment, were uniparous or multiparous, nonpregnant, and between 7 and 16 months of age.

All mice, with the exception of intact controls, were hypophysectomized-ovariectomized-adrenalectomized. Ovariectomy-adrenalectomy was performed 1 day prior to hypophysectomy. The surgical techniques and postoperative care have been described previously (*11*). Beginning on the day after hypophysectomy, one of the following hormone combinations was administered for 15 days: (*a*) deoxycorticosterone acetate (DCA), 125 μg/day + corticosterone acetate (B), 125 μg/day; (*b*) DCA, 125 μg/day + B, 125 μg/day + STH, 100 μg/day; (*c*) DCA, 125 μg/day + B, 125 μg/day + MH, 100 μg/day.

The hormones were administered subcutaneously in a volume of 0.2 ml, the steroids being combined in a single injection of 0.2 ml. The preparation of aqueous steroid suspensions and protein solutions has been described elsewhere (*11*). The purified ovine MH and bovine STH were generously provided by Professor C. H. Li.

The mice were killed 16 days after the start of hormone treatment. Each animal was carefully examined under a dissecting microscope for

possible remnants of ovaries, adrenals, and pituitaries; only those animals in which all operations were complete are included in the reported results.

Wholemount preparations of all 10 mammary glands were made from each animal. The state of development of the normal mammary-gland parenchyma, as well as the morphology of the hyperplastic nodules, was noted.

RESULTS

The various groups of control and experimental animals, with the number of mice used and the results obtained, have been summarized in tables 1 and 2.

In these experiments, the mice were not necessarily of the same age or breeding experience, since we are concerned only with the maintenance or regression of nodules after various hormonal treatments. Consequently, we have considered only the morphologic state of the nodules observed rather than their incidence.

Intact Control Mice

To study the morphology of normally occurring hyperplastic alveolar nodules, we killed several intact mice of each strain as controls. The development of ductal and alveolar elements in the normal mammary-gland parenchyma was variable within a single strain as well as among the several strains. In general, the mammary glands consisted of slender ducts with fine terminal branches and contained few or no alveoli.

Hyperplastic nodules were found in the mammary glands of most intact animals (figs. 1 through 5) in all strains (table 1). Morphologically, these nodules could be divided into the following categories:

1) *Well-maintained nodules.*—These were typical hyperplastic alveolar nodules consisting of closely packed clusters of alveoli, morphologically similar to the lobules of midpregnancy (fig. 1). In all strains, most nodules were of this type.

2) *Secretion-filled nodules.*—These were similar to the well-maintained nodules, except that the alveoli were more dilated and filled with serous secretion (fig. 2). A few nodules in all strains were of this type.

3) *Plaque-type nodules.*—These were like the secretion-filled nodules, but they were unusually large, several mm in diameter (fig. 4). The alveoli were even more dilated with serous secretion than those described in figure 2. A small number of nodules in C3H/2 and RIII mammary glands was of the plaque type.

4) *Semiregressed nodules.*—These were nodules with minute alvoeli (fig. 3). A small number of nodules in the glands of C3H and DBA mice was of this type.

5) *Ductal-type nodules.*—These nodules, found only in DBA females, were small, densely staining hyperplastic areas composed of a mass of

small, terminal budlike structures (fig. 1). Some nodules in DBA females were similar but contained a few alveoli scattered among the ductal elements.

6) *Inflammatory nodules.*—These were like the inflammatory nodules described by Huseby and Bittner (*12*). They were seen (fig. 5) only in A and A/3 females.

Mice Treated With DCA + B (Table 1)

Fewer terminal ducts were found in the mammary parenchyma of hypophysectomized-ovariectomized-adrenalectomized mice treated with DCA + B than in control animals of the same strain. In all strains, most of the hyperplastic alveolar nodules were in a regressed condition (figs. 6 through 11) after 15 days of hormone treatment. These regressed nodules, like the ductal-type nodules observed in intact DBA females, were composed of clusters of terminal ducts with few or no alveoli.

A few nodules in C3H, RIII, and DBA females were of the semiregressed type, whereas in C3H/2 and RIII mice some nodules composed of huge masses of ductal branches were presumably regressed plaque-type nodules. An occasional well-maintained nodule was seen in the glands of C3H, C3H/2, and DBA mice after DCA + B treatment.

Mice Treated With DCA + B + STH (Table 2)

The mammary parenchyma in hypophysectomized-ovariectomized-adrenalectomized mice of all strains treated with DCA + B + STH was similar to that of intact controls.

Although the majority of the nodules in all strains was well maintained (figs. 12 through 16), a small number of semiregressed nodules was present in the glands of all strains except RIII. Two of the 155 nodules seen in DBA females were completely regressed and resembled the ductal-type nodules described (*see* fig. 12).

At autopsy, many milk-filled nodules were observed in the mammary glands of RIII females. One milk-filled nodule also was seen in a DBA mouse, but there was no milklike secretion in any other strain.

Mice Treated With DCA + B + MH (Table 2)

The mammary parenchyma in hypophysectomized-ovariectomized-adrenalectomized mice of various strains treated with DCA + B + MH was similar to that of intact females.

Although most nodules (figs. 17 through 21) in all strains were well maintained after this treatment, there was a slightly higher number of regressed and semiregressed nodules among the C3H, C3H/2, and DBA females than in the same strains treated with DCA + B + STH. At autopsy, milk-filled nodules were observed only in RIII females.

TABLE 1.—Condition of hyperplastic alveolar nodules in intact and DCA- + B-treated hypophysectomized-ovariectomized-adrenalectomized mice of various strains

Strain	Intact					DCA- + B-treated mice*				
	Number of mice	Total number of nodules recorded	Types of nodules			Number of mice	Total number of nodules recorded	Types of nodules		
			Regressed	Semiregressed	Maintained†			Regressed	Semiregressed	Maintained†
C3H/Crgl	4	158	0	4	154	8	75	67	7	1
C3H/Crgl/2	8	220	0	0	220	7	50	49	0	1
RIII/DmCrgl	4	67	0	0	67	9	186	182	4	0
DBA/2NCrgl‡	6	270	0	16	254	10	99	92	4	3
A/Crgl	9	226	0	34	192	4	22	21	0	1
A/Crgl/3	6	25	0	0	25	5	4	4	0	0

*See text for doses of hormones used.

†Consists of total of well-maintained nodules, secretion-filled nodules, and plaque-type nodules. See text for description.

‡Total number of nodules does not include 6 ductal nodules observed.

TABLE 2.—Effect of various hormonal treatments on the condition of hyperplastic alveolar nodules of hypophysectomized-ovariectomized-adrenalectomized mice of various strains

Strain	Treatment: DCA + B + STH*					Treatment: DCA + B + MH*				
	Number of mice	Total No. of nodules recorded	Types of nodules			Number of mice	Total No. of nodules recorded	Types of nodules		
			Regressed	Semiregressed	Maintained†			Regressed	Semiregressed	Maintained†
C3H/Crgl	5	21	0	1	20	5	21	10	3	8
C3H/Crgl/2	7	221	0	2	219	7	89	6	6	77
RIII/DmCrgl	4	240	0	0	240	6	305	0	0	305
DBA/2NCrgl	6	167	2	10	155	5	127	5	13	109
A/Crgl	7	79	0	3	76	4	60	0	12	48
A/Crgl/3	5	2	0	0	2	5	1	0	0	1

*See text for doses of hormones used.

†Consists of total of well-maintained nodules, secretion-filled nodules, and plaque-type nodules. See text for description.

DISCUSSION

The spontaneous incidence of mammary cancer in the various strains of mice used in this study has been reported previously (*5*). In general, C3H, RIII, and DBA mice have a high incidence of mammary tumors in both virgin and parous females. The A and A/3 mice generally develop tumors only when parous, and the C3H/2 strain has a low incidence of mammary tumors, even among the breeding females.

Bittner (*13*) postulated that the development of mammary cancer in mice was dependent upon three factors: genetic predisposition, adequate hormonal stimulation, and the presence of the mammary-tumor agent. The low incidence of tumors in our C3H/2 subline is presumably due to the disappearance of the mammary-tumor agent (Barnawell: Unpublished observations).

Huseby and Bittner (*12*) believed that the same three factors were responsible for the appearance of hyperplastic alveolar nodules in the mammary glands. However, more Jones (*14*) found a large number of nodules in mice from a high tumor strain that had been made free of the mammary-tumor agent by foster nursing. In the present study, we have found hyperplastic nodules in all strains examined, including the supposedly agent-free C3H/2 mice.

The morphology of hyperplastic alveolar nodules in intact mice has been reported in detail (*12*, *15–20*). We found that well-maintained nodules were predominant in the strains used herein. Plaque-type nodules were found in small numbers and only in C3H/2 and RIII mice,[4] though Foulds (*21*) found plaquelike structures the most numerous in various hybrid mice that he studied. The ductal-type nodules observed here in the DBA strain were similar to those described by Pullinger (*18*) in RIII mice, and were composed of small ducts with terminal buds. These differed from the ductal nodules seen in strain A animals by Huseby and Bittner (*12*), which consisted of finely branching terminal ducts.

Other morphologic types of nodules were seen, such as semiregressed and secretion-filled nodules. Sometimes more than one type occurred in a single animal, which indicated that the hormonal requirements for the maintenance of these nodules are variable. The differences in sensitivity of the various nodule types have been discussed previously (*2*, *3*, *7*).

The presence of nodules in the mammary glands of nonpregnant, parous females indicates that nodules can be maintained by the hormones present even in such nonpregnant mice. In our recent studies in C3H animals, most nodules (*2*, *3*) were found to regress after hypophysectomy, and from the data reported here, it appears that adrenocortical steroids (DCA + B) cannot prevent the regression of the majority of nodules after hypophysectomy-ovariectomy-adrenalectomy in any strain studied. Most nodules were maintained, however, when either DCA + B + STH

[4] Recently we have encountered in multiparous C3H/Crgl mice an occasional large plaque closely resembling those described by Foulds (*21*).

or DCA + B + MH was administered to these mice. No significant difference in nodule-maintaining ability was observed between the two pituitary hormones.

The studies of Mühlbock (*6*) and Mühlbock and Boot (*22*) indicate that neither progesterone nor MH is present in significant quantities in nonpregnant mice. However, adrenocortical hormones and STH are undoubtedly present and can account for the maintenance of hyperplastic alveolar nodules in the mammary glands of intact, nonpregnant females.

Hyperplastic nodules in the mouse mammary glands are considered to be precancerous lesions, though this has been established incontrovertibly only for the well-maintained nodules of the C3H/Crgl mouse (*8*). Unlike the majority of nodules, most mammary tumors do not require pituitary hormones for their maintenance (*17*, *23*). A few nodules, however, also appear to be independent of pituitary hormones. In a study in which primarily pregnant hybrid mice were used, Gardner (*17*) reported that some nodules did not disappear after hypophysectomy. We also have found that some nodules fail to regress after hypophysectomy, but no correlation was found between the size or type of nodule and this apparent hormone independency. Bladder-like nodules, possibly consisting of cystically dilated ducts containing inspissated secretion, also appear to remain occasionally in C3H/Crgl mice after hypophysectomy (*7*, *20*). All the plaques, which were the largest nodules observed, regressed in hypophysectomized-ovariectomized-adrenalectomized animals treated only with DCA + B.

Although most nodules require some pituitary hormone for their maintenance, nodules in all mouse strains used responded similarly to MH and STH. In mouse strains that show a high incidence of mammary tumors in both virgin and breeding females (C3H, RIII, and DBA), we have shown that STH and MH are equally effective in the production of lobuloalveolar development and also in lactogenesis (*5*). In contrast, in the A and A/3 mice, which develop mammary tumors only in parous females, STH-containing combinations were found ineffective in combined lobuloalveolar mammogenesis and lactogenesis. MH was found to be lobuloalvelar mammogenic and lactogenic in all strains, when given with appropriate steroid hormones.

Our work with the C3H/Crgl strain has indicated that hormonal combinations which induce lobuloalveolar development also induce nodule development. Similarly, nodule-maintaining combinations also permit the neoplastic development of the nodules (*3*, *4*, *24*). From the results reported here, it appears that the genetic background of the mouse strain determines the lobuloalveolar mammogenic and probably the noduligenic response to somatotropin. Once the nodules are formed, no strain differences appear to exist in their sensitivity to pituitary hormones. Thus, virgin mice that do not develop tumors (*e.g.*, strains A and A/3) probably fail to do so because the hyperplastic alveolar nodules do not develop in response to the hormones present in the nonpregnant animal. However, once the nodules are formed in these strains, no

further subjection to pregnancy hormones is needed for their maintenance, and some tumors can be expected to arise.

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PLATES

Figures in plates show wholemounts of mammary glands from parous, non-pregnant female mice of various strains. All wholemounts were stained with hematoxylin and photographed at × 6, with the exception of figures 18 and 24, which were photographed × 16.

PLATE 25

FIGURE 1.—Untreated intact DBA/2NCrgl. *Note* presence of well-maintained nodules (MN) and ductal nodules (DN).

FIGURE 2.—Untreated intact RIII/DmCrgl. *Note* presence of secretion-filled nodule (SEN).

FIGURE 3.—Untreated intact C3H/Crgl. *Note* presence of semiregressed nodule (SN).

FIGURE 4.—Untreated C3H/Crgl/2. *Note* large, secretion-filled plaque-type nodule (*arrow*).

FIGURE 5.—Untreated intact A/Crgl. *Note* presence of 2 inflammatory nodules (*arrows*).

FIGURE 6.—Hypophysectomized-ovariectomized-adrenalectomized DBA/2NCrgl, treated daily for 15 days with DCA + B. *Note* regressed nodule (RN) with no recognizable alveoli.

FIGURE 7.—Hypophysectomized-ovariectomized-adrenalectomized RIII/DmCrgl, treated daily for 15 days with DCA + B. There are several RN's.

FIGURE 8.—As in figure 7. *Note* presence of large RN and a large SN.

FIGURE 9.—Hypophysectomized-ovariectomized-adrenalectomized C3H/Crgl, treated daily for 15 days with DCA + B. *Note* the similarity of RN to those in figures 6, 7, and 8.

FIGURE 10.—Hypophysectomized-ovariectomized-adrenalectomized C3H/Crgl/2, treated daily for 15 days with DCA + B. *Note* peculiar RN (*arrow*) characteristic of this subline and compare with RN in C3H/Crgl line (fig. 9).

FIGURE 11.—Hypophysectomized-ovariectomized-adrenalectomized A/Crgl, treated daily for 15 days with DCA + B. *Note* presence of 2 RN's.

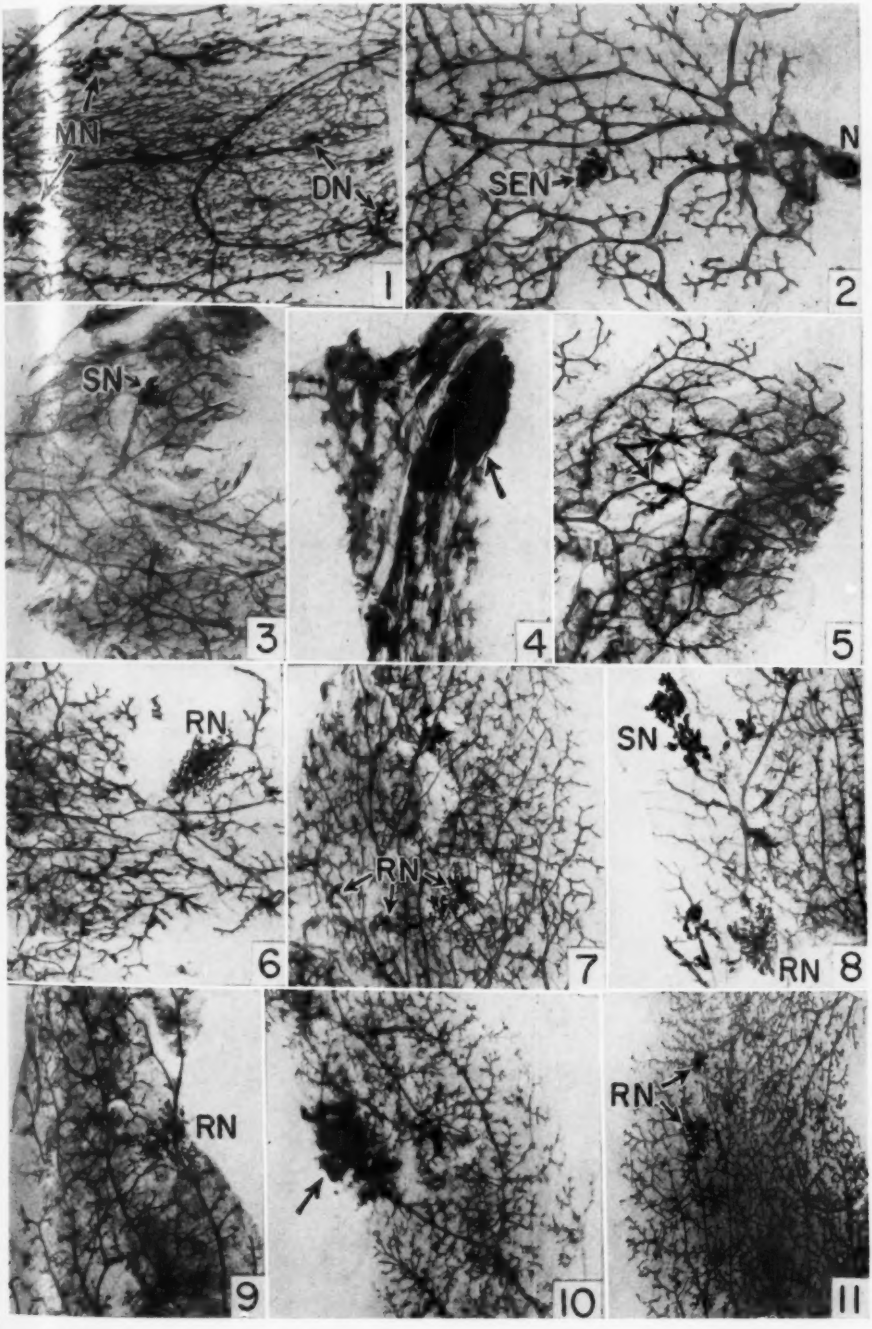

MN
DN
1
SEN
N
2
SN
3
4
5
RN
6
RN
7
SN
RN
8
RN
9
10
RN
11

PLATE 26

Figures in this plate are photographs of mammary glands from hypophysectomized-ovariectomized-adrenalectomized female mice treated daily with DCA + B + STH or with DCA + B + MH for 15 days.

FIGURE 12.—DBA/2NCrgl, treated with DCA + B + STH. *Note* SEN and also a DN or RN (*arrow*).

FIGURE 13.—RIII/DmCrgl, treated with DCA + B + STH. *Note* large plaque-type SEN.

FIGURE 14.—C3H/Crgl, treated with DCA + B + STH. *Note* SEN's.

FIGURE 15.—C3H/Crgl/2, treated with DCA + B + STH. *Note* SEN and several MN's.

FIGURE 16.—A/Crgl, treated with DCA + B + STH. *Note* SEN and several MN's. Figure 16a shows SEN from figure 16 at higher magnification. × 16

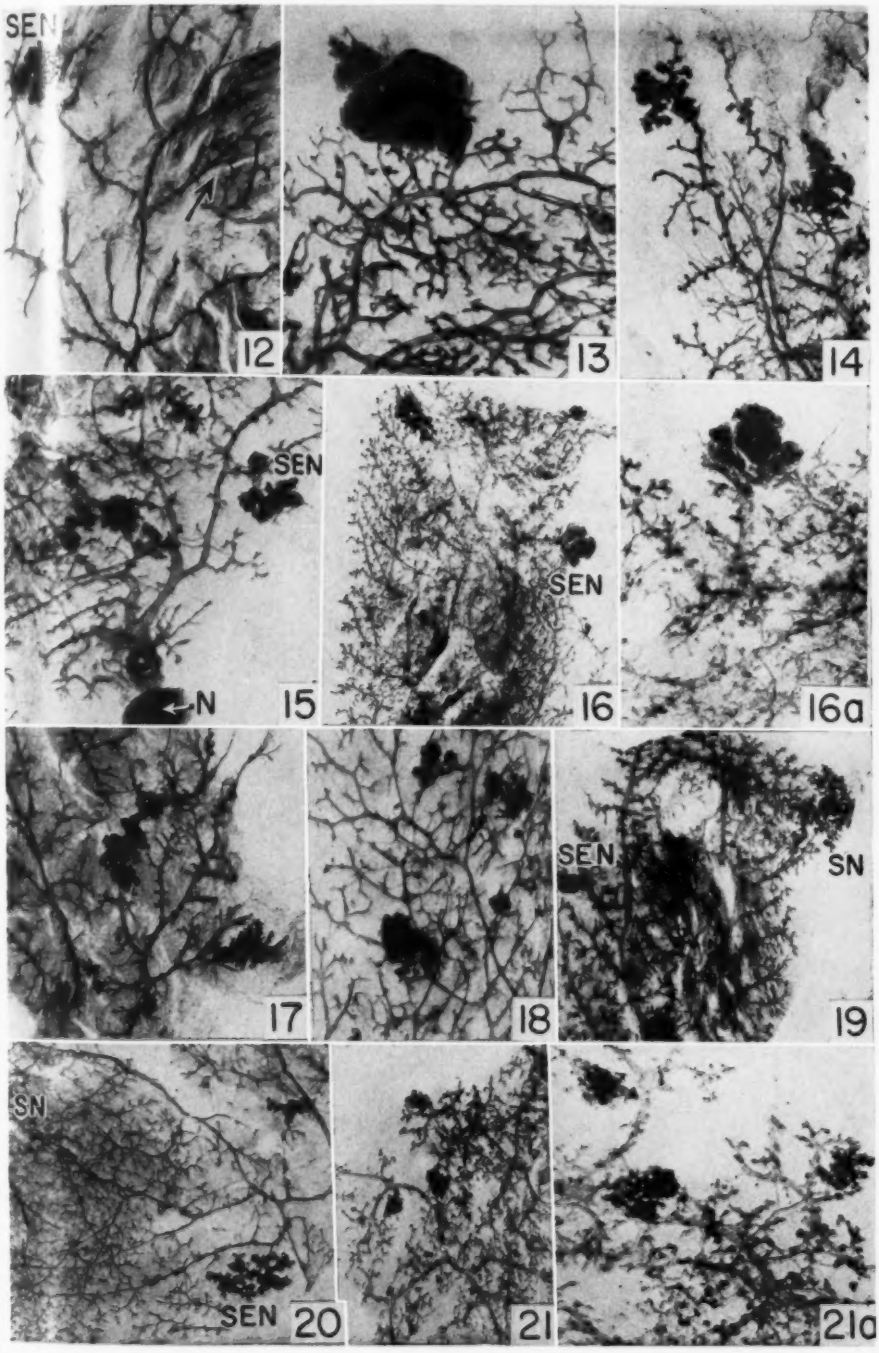
FIGURE 17.—DBA/2NCrgl, treated with DCA + B + MH. There are several SEN's.

FIGURE 18.—RIII/DmCrgl, treated with DCA + B + MH. There are several SEN's.

FIGURE 19.—C3H/Crgl, treated with DCA + B + MH. *Note* presence of SEN and SN.

FIGURE 20.—C3H/Crgl/2, treated with DCA + B + MH. There is an SEN as well as an SN.

FIGURE 21.—A/Crgl, treated with DCA + B + MH. *Note* SEN's. Figure 21a shows these nodules at higher magnification. ×16


SEN
12
13
14
SEN
N
15
SEN
16
16a
17
18
SEN
SN
19
SN
SEN
20
21
21a

Thresholds in Linear Dose-Response Models for Carcinogenesis [1]

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SUMMARY

This report shows how a curvilinear dose-response curve relating the number of tumors induced to the level of carcinogen employed can be reconciled with a single-stage model (insofar as the carcinogen is concerned) for carcinogenesis. This is an extension of the results of Heston and Schneiderman who showed that the number of pulmonary tumors induced in mice by dibenz[*a*,*h*]anthracene was proportional to the amount by which the dosage exceeded some "threshold" level. In the present investigation, each animal is postulated to have its own characteristic "threshold" for the carcinogen and its own characteristic "sensitivity" (increase in number of tumors per unit-dose increase). Under rather broad conditions it is demonstrated, and some hypothetical instances are given, that at sufficiently high doses there will be a linear relationship between the number of tumors per animal and the dosage used. At the lower levels curvilinearity results from the variation between individual "thresholds." How the curvilinear and the linear portions of the response curve are related to the patterns of individual variation in "threshold" and "sensitivity" and the intercorrelation of these two are described. Data on pulmonary-tumor induction in mice by dibenz-[*a*,*h*]anthracene (2 experiments) and urethan are presented. At the higher levels these show characteristic linear dose-response patterns. On the two occasions of testing, the carcinogenic activity of dibenz[*a*,*h*]anthracene differed by about a factor of 3, which may have been related to differences in particle-size distribution for the two preparations used. There was no significant difference in "thresholds" on these occasions when adjustment was made for the differences in activity. The activity of urethan was about 1 percent of that for dibenz[*a*,*h*]anthracene, and the activity-adjusted "threshold" was about the same as for dibenz-[*a*,*h*]anthracene.—J. Nat. Cancer Inst. 27: 203–215, 1961.

IN 1953, Heston and Schneiderman (*1*) published the results of an investigation of the mechanism whereby the carcinogen dibenz[*a*,*h*]anthracene induces pulmonary tumors in mice. This study was motivated by the earlier results of Charles and Luce-Clausen (*2*), who indicated that two

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events were involved in the change of a cell to form a papilloma. These would be necessary if the change were a recessive mutation. This interpretation was based on the demonstration, in mice painted repeatedly with benzpyrene, of a linear relationship between the square root of the number of papillomas resulting and the elapsed time, which would, in turn, be proportional to the cumulative dose of carcinogen.

In contrast to the repeated treatment procedure of Charles and Luce-Clausen, Heston and Schneiderman gave single injections of graded amounts of carcinogen. They showed that a linear relationship existed between the actual number of lung tumors induced and the amount of dibenz[*a*,*h*]anthracene injected. They interpreted this to mean that the compound was involved in only a single change in the cell that became neoplastic, and if the change were a gene mutation it would be a dominant mutation. This does not preclude the possibility that other changes not involving dibenz[*a*,*h*]anthracene might be necessary for tumor occurrence.

A feature of the Heston-Schneiderman study was that the straight-line relationship between the number of induced tumors and the carcinogenic dose was not one of simple proportionality: Rather, the number of tumors (over and above the expected spontaneous frequency) was proportional to the amount by which the dose exceeded some "threshold" level. Accordingly, while at high drug levels the linear relationship might continue to apply, a curvilinear relationship would obtain at very low treatment levels.

The purpose of our report is to show how both the early curvilinear portion and the subsequent straight-line relationship can arise. The curvilinear portion of the response curve can be simply reconciled with the subsequent linear relationship on the basis of variation in "thresholds." This requires only that each mouse have its individual ability to "dispose" of carcinogen, the expected number of tumors for the mouse being proportional to the dose in excess of its individual "threshold." The possibility that mice may vary also in their "sensitivities" to drug, *i.e.*, the factor of proportionality which may or may not be related to their "thresholds," is also explored.

Variation in both "sensitivity" and "threshold," whether correlated or uncorrelated, is investigated from a mathematical-statistical point of view, and the effects of such variation are illustrated for particular hypothetical cases. The results of the mathematical investigation provide a basis for the analysis of the data obtained in the tests of dibenz-[*a*,*h*]anthracene and urethan for their carcinogenic effects.

THE DOSE-RESPONSE MODEL

Our most general model is one in which each individual animal is characterized by its own "threshold" for and "sensitivity" to a carcinogenic agent. Let the "threshold" of the i'th animal be t_i, this being the lowest dose capable of inducing tumors in this animal. Further, let the

i'th "sensitivity" be b_i, this being the proportionality factor relating the expected number of tumors induced by a particular dose to the excess of that dose over the individual's threshold.

Let us represent the number of tumors elicited in the i'th animal by the dosage level X as $(Y_i|X)$. $(Y_i|X)$ is a random variable, and our model requires that its expectation be given by

$$E(Y_i|X) = b_i(X - t_i),\ X \geq t_i$$

and that

$$(Y_i|X) \equiv 0,\ X \leq t_i.$$

For convenience we may write

$$E(Y_i|X) \equiv -a_i + b_iX,\ X \geq t_i$$

where $a_i = b_it_i$, and may be thought of as the "tumor threshold," the threshold of the i'th individual expressed in units of number of tumors rather than in dose units.

The following is a discussion of the effects of variation from animal to animal in their t_i, b_i, and a_i values. The chance variation in $(Y_i|X)$ for $X > t_i$ is not germane to the development, but a reasonable law for such variation is that the number of induced tumors follow a Poisson distribution. The fact that an individual's threshold has been exceeded does not imply that any tumors will be induced, but only that there is a chance for the induction of tumors.

Population-Response Curve

The two expressions given [$(Y_i|X) = 0,\ X \leq t_i$, and

$$E(Y_i|X) = b_i\,(X - t_i) = -a_i + b_iX,\ X \geq t_i]$$

may be thought of as being the individual-response curve for the i'th animal. They describe what, on the average, will be the number of tumors elicited in a particular animal by a particular dose.

The population-dose-response curve is the average of all the individual response curves in the population. Conceptually, it relates the average number of tumors elicited in an animal chosen at random from the population to the dose. If we represent the number of tumors arising in an animal selected at random as $(Y|X)$, the population-dose-response curve can be represented as $E(Y|X)$. And as the individual-response curve, $E(Y_i|X)$, depends on the individual t_i and b_i or a_i and b_i values, the population-response curve will depend on all the sets of t_i and b_i or a_i and b_i values in the population.

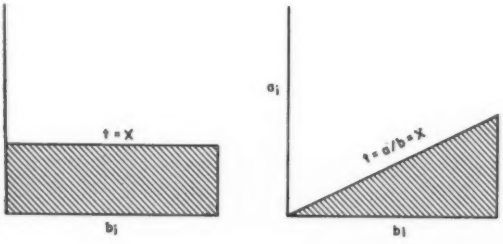
Symbolically, the population-response curve can be expressed as

$$E(Y|X) = P_{(x)}\,[\overline{b}_{(x)}X - \overline{bt}_{(x)}]$$

$$= P_{(x)}\,[\overline{b}_{(x)}X - \overline{a}_{(x)}],$$

where $P_{(x)}$ is the proportion of individuals with "threshold" or t_i values less than X; $\overline{b}_{(x)}$ and $\overline{bt}_{(x)} = \overline{a}_{(x)}$ are, respectively, the average b_i and $b_i t_i = a_i$ values of individuals with "thresholds" less than X.

The two panels of text-figure 1 show these concepts graphically. We may suppose that each individual has characteristic t_i and b_i values, both non-negative, and that these define a bivariate distribution over the plane. The hatched area in the *left panel* corresponds to the region in which the "threshold" value, t_i, is less than some particular X. The proportion of individuals whose t_i and b_i values fall in this region is $P_{(x)}$; the average value of b_i in this region is $\overline{b}_{(x)}$, while the average value of $b_i t_i$ is $\overline{bt}_{(x)}$.



TEXT-FIGURE 1.—Illustration of regions corresponding to "thresholds," t, less than some particular dose, X. The proportion of individuals whose t and b values (*left panel*) or a and b values (*right panel*) fall within the hatched areas defines $P_{(x)}$. The average values of b and $a = bt$ for such individuals define $\overline{b}_{(x)}$ and $\overline{a}_{(x)} = \overline{bt}_{(x)}$. *See* discussion in text.

The *right panel* shows the bivariate distribution in terms of the a_i and b_i values. Since $t_i = a_i/b_i$, differing t values are defined by lines through the origin with differing slopes. Again the hatched area, this time defined by a diagonal line, corresponds to the region in which the "threshold" value is less than X, and again $P_{(x)}$, $\overline{b}_{(x)}$, and $\overline{a}_{(x)} = \overline{bt}_{(x)}$ are defined over this region.

Some Considerations Relative to Population-Response Curve

Some results arise immediately upon consideration of the population-response curve

$$E(Y|X) = P_{(x)}[\overline{b}_{(x)}X - \overline{bt}_{(x)}] = P_{(x)}[\overline{b}_{(x)}X - \overline{a}_{(x)}].$$

That this response curve is monotone and increasing with dose is clear. It may be nondecreasing over the region in which $E(Y|X) = 0$. Suppose that all the "threshold" values, t_i, are bounded and less than or equal to some t_{max}. Then for $X > t_{max}$, $P_{(x)} = 1$, and $\overline{b}_{(x)}$ and $\overline{bt}_{(x)} = \overline{a}_{(x)}$ will equal their population values, $\overline{b}$ and $\overline{bt} = \overline{a}$, respectively. The response curve for $X > t_{max}$ will then be given by

$$E(Y|X) = \overline{b}X - \overline{bt} = \overline{b}X - a,$$

a straight line. The population-response curve, though curvilinear in the early portion of the curve, eventually becomes a straight line. This result would still apply even if some proportion of individuals were immune, their "thresholds" unbounded. Then $\bar{b}$, $\overline{bt}$, and $\bar{a}$ must only be defined relative to the nonimmune, and the expression for $E(Y|X)$ would require multiplication by the proportion of nonimmunes in the population.

It also follows that the curvilinear portion of the population-response curve is characterized by a nondecreasing slope, concave upward with a slope gradually increasing to that obtaining in the linear portion. This comes about because the slope at any value of X on the curve is proportional to the sum of the "sensitivity" or b values of individuals with "thresholds" less than X. While the average "sensitivity" of individuals with "thresholds" less than X may or may not increase with X, the sum of such "sensitivities" is necessarily a nondecreasing function of X.

If, to avoid the effects of curvature in the low dose range, data are secured at dose levels in excess of virtually all the individual "thresholds," the straight lines fitted by appropriate methods will tend to duplicate the population-response-curve straight line. If we assume no immunes in the population, the slope of the fitted line will thus tend to equal $\bar{b}$, the population average "sensitivity." The negative of the intercept of the fitted line will tend to equal $\bar{a}$, the population average "tumor threshold." But $\bar{a} = \overline{bt} = \bar{b}\,\bar{t}$ + covariance (b, t), indicating that the ratio of the negative of the intercept to the slope (this is the X-intercept) does not, in general, tend to equal the average "threshold," except when there is no correlation between individual "threshold" and "sensitivity" values. A positive correlation makes the ratio or observed X-intercept an overestimate of the average "threshold," while a negative correlation makes it an underestimate.

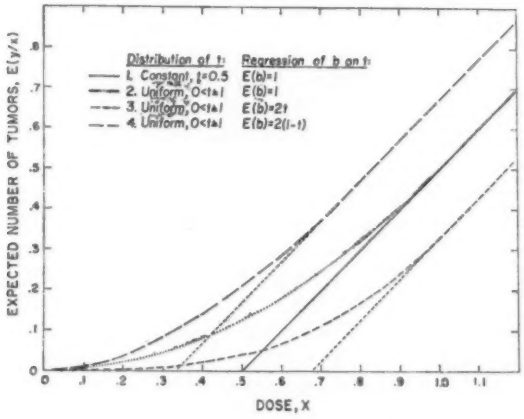
In particular, there is no correlation between "threshold" and "sensitivity" when either all "thresholds" or all "sensitivities" are uniform. If all "thresholds" are uniform, whether or not the "sensitivities" vary, the early curvilinear portion of the response curve will disappear. With uniform "sensitivities" but varying "thresholds," the curvilinear portion will remain. In either case the average "threshold" is given by the negative of the intercept to slope ratio.

These concepts will be illustrated with hypothetical situations involving positively and negatively correlated variation in "threshold" and "sensitivity." Finally, experimental results obtained with dibenz[a,h]anthracene on two separate occasions and urethan on one will be analyzed from the points of view expressed.

ILLUSTRATIONS AND APPLICATIONS

Table 1 and text-figure 2 illustrate the concepts described with 4 hypothetical examples involving variation in individual "thresholds" or

"sensitivities." The examples are comparable in that for each, the average "threshold" is 0.5, with no individual "thresholds" in excess of unity, and the average "sensitivity" is unity. The various measures describing the relationship between expected response in number of tumors and dose level are shown in table 1 separately for dose levels both below and above that corresponding to the maximum "threshold," while text-figure 2 shows the population-response curves in graphic form.



TEXT-FIGURE 2.—Dose-response curves illustrative of hypothetical examples given in table 1.

The first example is one in which all individual "thresholds" are constant at 0.5 while the "sensitivity" is permitted to vary around an average of 1. Table 1 specifies uniform variation between 0 and 2; however, for the case of constant thresholds the population-response curve is not altered by the law of variation in "sensitivity," only by the average "sensitivity." For this example the population-response curve changes abruptly at dose level 0.5 from one in which no tumors are elicited to one in which the expected response is proportional to the amount by which the dose exceeds 0.5. Data obtained with dose levels in excess of 0.5 would correctly suggest that the average "threshold" was 0.5, the average "sensitivity," 1.0.

In the next 3 examples the "threshold" is permitted to vary uniformly between 0 and 1, with an average of 0.5, and different relationships between "sensitivity" and "threshold" are considered.

For example 2, "sensitivity" and "threshold" are assumed to be unrelated. Though the "sensitivity" is shown constant at unity, the same result would apply if variation were allowed just so long as, on the average, individuals with differing "thresholds" had the same "sensitivity." In this example the population-response curve shows a smooth transition at $X = 1.0$ from a curve to a straight line. Data obtained at

TABLE 1.—Hypothetical examples involving variation in individual "thresholds" or "sensitivities"

Distribution of "threshold," t	Constant, $t = \frac{1}{2}$		Uniform: $0<t\leq 1$					
Distribution of "sensitivity," b	Uniform: $0<b<2$		Constant, $b = 1$		Not specified			
Regression of b on t	$E(b) = 1$		$E(b) = 1$		$E(b) = 2t$		$E(b) = 2(1-t)$	
Covariance of b and t	0		0		$\frac{1}{6}$		$-\frac{1}{6}$	
Dose level considered, X	$X<\frac{1}{2}$	$X\geq\frac{1}{2}$	$X<1$	$X\geq 1$	$X<1$	$X\geq 1$	$X<1$	$X\geq 1$
For individuals with "threshold" $< X$:								
$\bar{t}_{(x)}$, average, "threshold"	0	1/2	$X/2$	1/2	$X/2$	1/2	$X/2$	1/2
$\bar{b}_{(x)}$, average "sensitivity"	0	1	1	1	X	1	$2-X$	1
$P_{(x)}$, proportion of population	0	1	X	1	X	1	X	1
$\overline{bt}_{(x)} = \bar{a}_{(x)}$, average "tumor threshold"	0	1/2	$X/2$	1/2	$\frac{2X^2}{3}$	2/3	$X-\frac{2X^2}{3}$	1/3
$E(Y\|X)$, expected number of tumors as function of dose	0	$X-1/2$	$\frac{X^2}{2}$	$X-1/2$	$\frac{X^3}{3}$	$X-2/3$	$X^2-\frac{X^3}{3}$	$X-1/3$
$\bar{a}_{(x)}/\bar{b}_{(x)}$, X-intercept of lines tangent to $E(Y\|X)$ (apparent "threshold" for $X>1$)	0	1/2	$X/2$	1/2	$2X/3$	2/3	$\frac{3X-2X^2}{3(2-X)}$	1/3

levels in excess of 1.0 would correctly suggest the average "threshold" to be 0.5, the average "sensitivity," 1.0.

In the 2 final examples, while the distributions of individual "sensitivities" are not identified, two rules for variation with "threshold" are given. In example 3, "sensitivity" is postulated to increase with "threshold," while in example 4 it decreases. In both cases the population-response curve shows a smooth transition at $X = 1.0$ from a curve to a straight line. While the average "sensitivities" of 1.0 would be correctly suggested in both instances by data obtained at sufficiently high dose levels, it can be seen that in example 3 the average "threshold" would be overestimated at ⅔, while in example 4 it would be underestimated at ½.

These 4 examples illustrate the point that above the maximum individual "threshold" the population-response curve follows a straight line with a slope corresponding to the population average "sensitivity." Where there is no correlation between "sensitivity" and "threshold," which is true for examples 1 and 2, the population average "threshold" is correctly suggested by the straight-line portion of the curve. If "sensitivity" tends to increase with "threshold," as in example 3, the population-average "threshold" is overestimated by the X-intercept corresponding to the straight-line portion of the curve. This would seem an unlikely possibility. "Sensitivity" more likely decreases with increasing "threshold," example 4, in which the population average "threshold" is underestimated by the straight-line X-intercept.

In all cases, the straight-line portion provides a correct estimate of the average "tumor threshold," $\bar{a}$, the average product of individual "sensitivity" and individual "threshold."

Application to Experimental Results [4]

The results of 2 experiments in table 2, which explore the pulmonary-tumor response of strain A mice to graded doses of dibenz[*a*,*h*]anthracene injected intravenously, are analyzed in relation to the model described. Experiment 1 is the same as that reported by Heston and Schneiderman (*1*); experiment 2 was performed subsequently to investigate the dose-response relation at low doses, but with a new preparation of dibenz[*a*,*h*]-anthracene.

Inspection of the data suggests that all the dose levels of experiment 1—the lowest, 0.1 mg per mouse—were in the linear portion of the response curve, while in experiment 2 dose levels of 0.02 mg per mouse and higher were in the linear portion. (Note that not all mice at the 0.02 mg level developed tumors; nevertheless, virtually all individual "thresholds"

[4] In all experiments described here, strain A mice received graded single injections of drug—colloidally dispersed dibenz[*a*,*h*]anthracene or dissolved urethan in 0.5 ml distilled water—control mice receiving injections of distilled water only. Mice, males and females, were about 2 months of age, the sexes being approximately equally divided in all experimental groups. Animals were individually identified, kept in plastic cages with 8 to a cage, fed Derwood pellets, and given an unlimited supply of tap water. Except for a few mice dying shortly after urethan injection, animals survived 6 months after injection when they were killed. Their fresh lungs were examined with the dissecting microscope, and the number of tumors appearing on the surface of the lungs of each mouse was recorded. At 6 months the lung tumors so induced in strain A mice were benign adenomas.

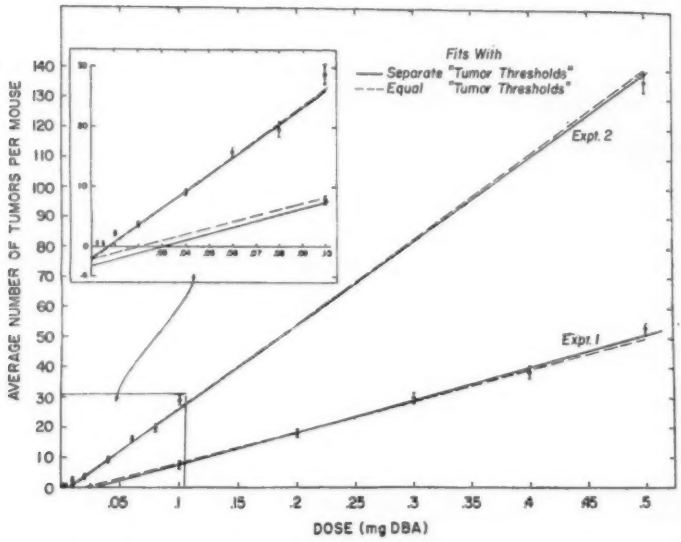
TABLE 2.—Pulmonary-tumor-response data

Carcinogen	Dose (mg)	Number of animals receiving injections	Percent response	Average No. nodules	Standard error of average
Dibenz[*a*,*h*]anthracene					
Experiment 1	0. 0	55	27	0. 29	0. 033
	0. 1	51	100	8. 08	0. 542
	0. 2	50	100	18. 25	1. 225
	0. 3	44	100	30. 02	1. 663
	0. 4	50	100	38. 64	1. 923
	0. 5	46	100	53. 37	2. 166
Experiment 2	0. 0	101	39	0. 46	0. 065
	0. 0025	48	60	0. 98	0. 170
	0. 005	44	70	0. 91	0. 168
	0. 01	52	86	2. 77	0. 353
	0. 02	98	93	4. 09	0. 248
	0. 04	54	100	9. 52	0. 685
	0. 06	55	100	16. 25	0. 837
	0. 08	54	100	20. 07	1. 325
	0. 1	55	100	29. 22	1. 480
	0. 5	50	100	136. 42	3. 641
Urethan	0. 0	38	24	0. 34	0. 109
	1. 25	39	74	1. 49	0. 179
	2. 5	36	86	2. 83	0. 312
	5	37	100	4. 65	0. 372
	10	39	100	11. 95	0. 798
	20	37	100	22. 65	0. 993
	40	22	100	35. 64	1. 860

could have been below that dose level.) Weighted least-squares, straight-line procedures, based only on results for dose levels apparently in the linear range, were used to compare the results obtained on the two occasions that dibenz[*a*,*h*]anthracene was tested. Weights employed in the least-squares analysis were the reciprocals of the squares of the standard errors corresponding to the empirical variation at each dose level. Data were adjusted for the frequency of spontaneous tumors, which were considered to have virtually no influence on the weighting factors. Table 3 and text-figure 3 summarize the statistical analysis made.

Text-figure 3 shows that there is substantially a linear relationship between numbers of pulmonary tumors and dibenz[*a*,*h*]anthracene dose levels at all but the very lowest levels of experiment 2. There is, however, a marked difference in the potency of dibenz[*a*,*h*]anthracene for eliciting tumors in the 2 experiments, as might have been expected since different preparations were used. In experiment 2, the "sensitivity" parameter, or slope, b, was almost 3 times as great as in the first (*see* first 2 columns, table 3) but there was no significant difference in the "tumor threshold" parameter, or negative Y-intercept, a. The X-intercepts in the 2 experiments were also clearly different, being only about one third as great in the second experiment as in the first, this reflecting principally the difference in "sensitivities."

The third and fourth columns of table 3 show a refitting of the data of the two experiments. This refitting reflects the fact that while both the



TEXT-FIGURE 3.—Pulmonary-tumor response to graded doses of dibenz[*a*,*h*]anthracene (DBA) in 2 experiments, with dose-response curves fitted under model of individual thresholds.

"sensitivity" and the "threshold" for an agent reflect its potency, the "tumor threshold" does not. If an agent of doubled potency (twice as concentrated) is used, the "sensitivity" will double and the "threshold" will halve, but the "tumor threshold" will remain unaltered. With the clear change in potency for dibenz[*a*,*h*]anthracene on the two occasions, and in view of the not significantly dissimilar "tumor thresholds," it became appropriate to fit the data under the assumption that the "tumor threshold" was constant. The comparison of the activities of dibenz-[*a*,*h*]anthracene in the two experiments remains substantially unaltered by the refit.

Since the same agent, dibenz[*a*,*h*]anthracene, was used in both experiments, the difference in potency observed needs to be accounted for. That the potency of dibenz[*a*,*h*]anthracene is influenced by particle size is known (*3*), and in the present instance, barring differences in animal susceptibility in the two experiments, it is likely that the dibenz[*a*,*h*]anthracene was milled to particles of larger average size in the second experiment.

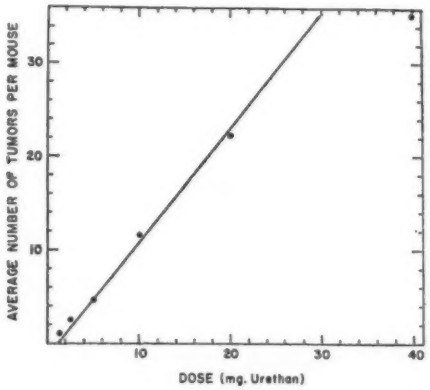
In view of the possibility that the observed tumor response to graded doses of dibenz[*a*,*h*]anthracene might reflect merely the distribution of particles of the compound in the lung from the intravenously injected dispersion of dibenz[*a*,*h*]anthracene crystals, data were obtained on pulmonary-tumor response of strain A mice to intraperitoneally injected urethan in solution. The results for a single experiment with urethan are shown in table 2, the statistical analysis is given in table 3, and the

TABLE 3.—Statistical analysis of pulmonary-tumor-response data

	Dibenz[a,h]anthracene				
	Separate intercepts		Common intercepts		
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Urethan
Dose range (mg)	0. 1–0. 5	0. 02–0. 5	0. 1–0. 5	0. 02–0. 5	1. 25–20
Number of spontaneous tumors	0. 29 ± 0. 033*	0. 46 ± 0. 065	0. 29	0. 46	0. 34 ± 0. 107
Tumor threshold: a	+3. 16 ± 0. 800	+1. 93 ± 0. 293	+2. 12 ± 0. 276		+1. 67 ± 0. 597
Sensitivity: b	108. 5 ± 4. 08	281. 3 ± 6. 54	104. 1 ± 2. 58	284. 0 ± 7. 37	1. 226 ± 0. 0686
X-intercept: $\frac{+a}{b}$	0. 029 ± 0. 0014	0. 0069 ± 0. 00028	0. 020 ± 0. 0019	0. 0074 ± 0. 00066	1. 362 ± 0. 0966

*± Indicates standard error.

graphic analysis in text-figure 4. The linear relationship between pulmonary-tumor incidence and dose extended from the lowest dose used, 1.25 mg/mouse, up to the next to highest dose, 20 mg/mouse, and it was over this range that the results were analyzed. The "sensitivity" parameter obtained for urethan in this experiment was from 0.4 to 1.2 percent that for dibenz[*a*,*h*]anthracene, according to whether comparison is made with the second or first dibenz[*a*,*h*]anthracene experiment. The "tumor threshold" measure of 1.67 tumors was not significantly different from that of 2.12 tumors observed in the refitting of the dibenz[*a*,*h*]anthracene data. Under these experimental conditions urethan appears to have a carcinogenic potency on the order of 1 percent that of dibenz[*a*,*h*]anthracene.



TEXT-FIGURE 4.—Pulmonary-tumor response to graded doses of urethan, with dose-response curve fitted under model of individual thresholds. (Response to 40 mg urethan not included in analysis.)

In the urethan experiment, the suggested deviation from linearity at the high dose of 40 mg is in the direction of a deficit in number of pulmonary tumors. This is not the direction that such a deviation would take if the carcinogenic role of urethan were multistage in character. A two-stage role, for example, would require that tumor incidence increase with the square of dosage (in excess of threshold) so that deviations from linearity at high doses would be positive. The moderate negative departure at the dose of 40 mg per mouse, if not due to some extreme chance variation, could perhaps be attributable to competition between or merging of pulmonary tumors when their numbers are large. This dose was therefore not included in the statistical analysis.

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Microspectrophotometric Determination of Deoxyribonucleic Acid in Primary and Metastatic Mouse Mammary Tumors [1]

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SUMMARY

A microspectrophotometric study of the relationship of ploidy to metastasis is described. Five spontaneous and 11 metastatic mouse mammary tumors were used. The 11 metastatic tumors included 9 intravenously transplanted and 2 spontaneous. All the metastatic tumors tended to have a higher ploidy than the primary tumors from which they were derived. Diploid cells, although present in the primary tumors, were absent from some metastases. However, the presence of diploid cells in some metastatic tumors suggested that diploid as well as polyploid cells were able to leave the primary tumor and establish themselves at metastatic foci.—J. Nat. Cancer Inst. 27: 217–220, 1961.

THE PLOIDY in both human and animal tumors has been extensively studied (*1–5*), and it is generally accepted that the deoxyribonucleic acid (DNA) content of the tumor cells exhibits wide variation from diploid to polyploid class. However, very little work has been done relating this observed variation in ploidy to metastases of neoplastic cells.

In studies on human cancers Rabotti (*1*) noted that in cells of primary tumors the modal class extended from diploid to polyploid, while the metastatic tumor nuclei showed a wide range of polyploid class but lacked the diploid class. This observation suggested two interesting and related hypotheses: 1) Only polyploid cells leave the site of the primary tumor. 2) Although cells of all ploidy classes leave the primary tumor, only the polyploid cells survive at a metastatic site.

The present study, with an animal system, was undertaken to gain additional information about these hypotheses.

MATERIALS AND METHODS

The animals chosen for this study were female C3H/HeN mice. Five mice bearing spontaneous mammary tumors, 1 cm or larger in diameter,

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

were used as donors. Two of the 5 also had spontaneous lung metastasis.[3] To produce transplant metastases,[4] nine 6- to 8-week-old C3H/HeN female mice received injections in the tail vein of a tumor-cell suspension prepared from each spontaneous mammary tumor.

For intravenous injections 25 mg of the primary tumor tissue was finely minced with scissors in 6 ml of 0.85 percent saline. The suspended tumor fragments were then passed through a series of hypodermic needles of decreasing sizes from gauge 18 to 26. Several dozen counts, in a hemocytometer, of tumor cells in a suspension thus prepared varied from 1.4 to 2.3 million per ml.

Each animal received 0.25 ml (about 0.4–0.5 million cells) of this preparation. The animals were killed 4 weeks after intravenous injection, and transplanted metastases were found only in the lungs.

For the microspectrophotometric studies, a portion of the tumor and of the autologous spleen, to serve as control, were fixed in 10 percent formalin. The tissues were embedded in the same paraffin block and the sections mounted on the same slide. Spontaneous metastasis, when present, was prepared alongside the primary tumor in the same paraffin block. The sections were cut at 6 μ and stained by the Feulgen method. One hundred nuclei randomly selected from different parts of the tumor were measured for the microspectrophotometric determinations. Sections were made for Feulgen studies and comparable sections were cut and stained with hematoxylin and eosin for morphological studies.

The microspectrophotometric technique (*5*, *6*) with light of 546 mμ wavelength was used to get a relative value of Feulgen-stained DNA of a single nucleus. The concentration of absorbing material in the nucleus was obtained from the product of extinction and volume. The instruments and the methods used in this study were similar to those described by Swift (*7*). The average value of three measurements in different areas of each nucleus was used to decrease errors arising from variations in concentration of DNA in the single nucleus.

RESULTS AND DISCUSSION

Representative histograms of the distribution pattern of DNA in the population of cell nuclei of the primary and metastatic tumors (both spontaneous and transplanted) are shown in text-figure 1.[5] Microspectrophotometric values of the splenic lymphocytes varied little from animal to animal.

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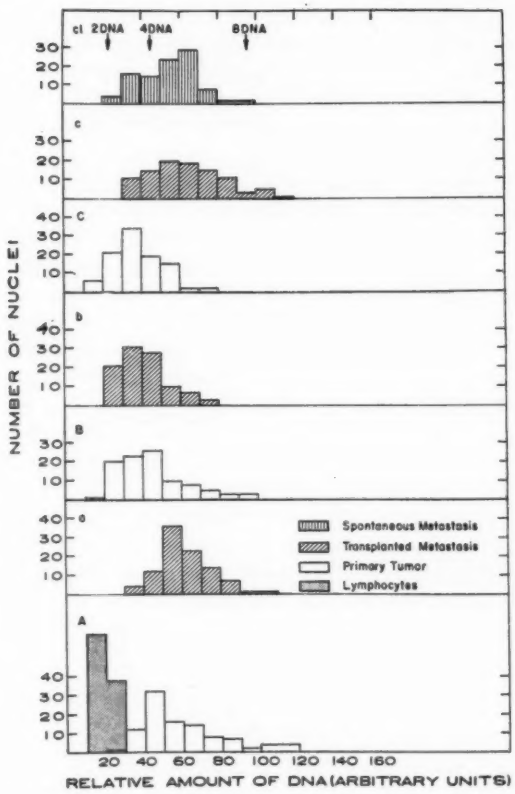
[3] "Spontaneous metastasis" refers to the foci of mammary-tumor tissue found in the internal organs of a mouse with a spontaneous mammary tumor.

[4] "Transplant metastases" refer to the foci of mamary tumor developing in the internal organs of mice receiving intravenous injections of a mammary-tumor cell suspension.

[5] In addition to 5 primary tumors, 2 spontaneous metastatic tumors, and 9 transplanted tumors, 8 transplanted metastases produced by intravenous injection of cells derived from previously produced transplanted metastases were examined. The histogram of these second-generation transplanted metastases uniformly showed a shift to the right toward the higher ploidy class.



TEXT-FIGURE 1.—Histograms of distribution patterns of DNA in population of cell nuclei of primary and metastatic tumors.

Histograms of the DNA content of the primary tumor nuclei showed a wide range of ploidy class. Although there was a tendency toward heteroploidy, the largest class of cells was tetraploid.

A comparison of the 3 primary tumors revealed considerable variation in the ploidy. This variation was particularly evident in the diploid class, where the number of cells varied from 1 percent in tumor A to 27 percent in tumor C.

The spontaneous metastasis contained cells of a slightly higher ploidy than the primary tumor from which it arose, but diploid cells were also present (Cl). A similiar pattern was seen in the transplanted metastases, for in tumor B the diploid class also persisted. In general, the histograms of both the spontaneous and metastatic tumors resembled more closely the histograms of the primary tumors from which they arose than they resembled each other.

Since the spontaneous metastatic tumor (Cl) contained cells of the diploid class, it is evident that diploid cells occurred in the metastases.

The presence of diploid cells in the transplanted metastases of tumor B confirmed this observation, though the absence of diploid cells in the transplanted metastases of tumors A and C is of interest. These observations would suggest that diploid, as well as polyploid, cells are able to leave the primary tumor and, transported via the blood stream, to establish themselves at a metastatic focus. The tendency for the transplanted metastasis to lack the diploid class as observed in tumors A and C, and as previously reported in human tumors (*1*), may well represent selection of the polyploid cells that may have the potential for more rapid self-reduplication. On the other hand, one cannot ignore the possibility that diploid cells may be transformed to polyploid cells in the metastatic site. On these bases, one might expect that the larger tumors would have fewer diploid cells. This is true of the transplanted metastatic tumors in this study, where tumor A measured 1.0 by 0.5 cm, tumor C, 0.3 by 0.3 cm, and tumor B, 0.1 by 0.2 cm.

In the assessment of these results it should be kept in mind that the transplanted metastasis occurs without the simultaneous presence of a primary tumor. Whether the coexistence of a primary and metastatic lesion influences the cell ploidy has not been shown by the present studies.

These observations indicate that, in the mouse mammary tumors examined, both diploid and polyploid cells are present in metastasis and suggest that diploid cells, as well as polyploid cells, are able to leave the primary tumor and establish themselves at metastatic foci.

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Intranuclear Inclusions in Tissue Cultures Infected With Rat Virus [1]

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SUMMARY

Rat virus (RV), originally isolated from tumor-bearing rats by Kilham, has many properties similar to those of polyoma virus. It has not been shown to have an oncogenic effect, however. This study was undertaken to determine the response of salivary gland of the rat to RV in tissue culture. Organotypic cultures of rat submandibular glands on gelatin sponge matrix were examined histologically at intervals from 10 to 32 days after infection with RV, and were compared with uninfected control cultures. Monolayer cultures of trypsinized rat embryo infected with RV were also examined. Type-A intranuclear inclusion bodies were found in epithelial as well as connective-tissue cells in the organotypic cultures at all time intervals under study. Similar inclusions were also found in cells of fibroblastic morphology in RV-infected monolayer cultures of rat embryo. The inclusions were similar to those previously observed in organotypic cultures of mouse salivary gland infected with polyoma virus, but tended to be larger and more eosinophilic. The response to RV was predominantly cytolytic and lacked the proliferative aspect that is characteristic of the response of epithelium of mouse salivary gland infected with polyoma virus. Necrosis of connective-tissue cells in RV-infected cultures resulted in disruption of the organoid structure of explants, with regrouping of epithelium in atypical patterns. Macrophage populations were decreased in RV-infected cultures. The observations are consistent with the apparent, but not fully proved, lack of oncogenic activity of RV on rat salivary gland *in vivo*.—J. Nat. Cancer Inst. 27: 221–235, 1961.

RAT VIRUS (RV) and the polyoma virus of mice have a number of properties in common. These have been discussed in an account of the isolation of rat virus from tumor-bearing rats (*1*). They include an ability to produce hemagglutination of guinea-pig erythrocytes by attachment to viral receptors that are removed by receptor-destroying enzyme, an inability to bring about hemadsorption in tissue cultures, and resistance both to ether and to relatively high temperatures. No serologic cross-

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[5] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

reactions have been demonstrated between the two agents. There is no positive evidence that RV is oncogenic in rats, but a clear test for oncogenic action has been precluded by the almost universal presence in rats of immunity to the agent, demonstrable by hemagglutination-inhibition (HI) tests in the serums of wild, laboratory, and germ-free strains (*1*).

Circumvention of this immunity is essential to the study of the pathologic effects of large inoculums of RV into newborn or adult rats. It is particularly important to know whether RV is capable, as is the polyoma virus, of inducing salivary-gland tumors. With the polyoma virus, it has been shown that the response of mouse salivary gland *in vitro* is similar in many respects to that *in vivo* (*2, 3*). The work reported here was initiated to determine whether rat salivary-gland tissue would respond to RV *in vitro* in a manner analogous to the response of mouse salivary-gland tissue to polyoma virus *in vitro*.

MATERIALS AND METHODS

The virus used was RV-13, which had been passed 12 times in rat-embryo tissue cultures. The hemagglutination (HA) titer of the final fluid pool was 1:640.

Two separate sets of gelatin sponge-matrix cultures, each including uninfected controls, were prepared. The virus preparation used to infect the first set was untreated, while that for the second set was heated at 65° C for 15 minutes to kill heat-sensitive extraneous viruses that might be present.

The culture technique was an adaptation of the Leighton sponge-matrix method (*4*), previously used for the polyoma-virus system (*2*). Submandibular glands removed aseptically from 2 Fischer rats, 10 and 22 days old, provided the tissue for the 2 sets of cultures. Explants cut to approximately 1.0 mm in diameter were mixed, and while half were allowed to stand in the virus preparation for 2 hours at room temperature, the other half stood in the culture medium alone for the same time before being placed in culture. Six to 8 explants were placed on the surface of a strip of gelatin sponge in each Leighton tube. No plasma clot was used.

The culture medium was made up of 30 percent pooled adult human serum and 70 percent Morgan, Morton, and Parker's mixture 199, with 100 units of penicillin per ml and 0.001 percent phenol in final concentration. Fluid volumes were maintained at approximately 1.0 ml per tube, with replacement of 0.75 ml of medium 2 to 3 times per week.

HA tests were performed, as elsewhere described (*1*), with guinea-pig erythrocytes used at room temperature. Six infected and 6 uninfected cultures were prepared in each of the 2 sets. Serial histologic sections were prepared from the first set as follows: 2 test and 2 control cultures at 18 days, and 4 test and 4 control cultures at 32 days. From the second set, serial sections were prepared from 3 test and 3 control cultures at 10

days, and from 1 test and 1 control culture at 24 days. The remaining cultures from this set were transplanted subcutaneously in newborn Fischer rats, but the recipients were lost by accident. Fixation was in Zenker-formol solution, and the sections were stained by hematoxylin and eosin. Alternate slides from the second set of cultures were left unstained until the hematoxylin and eosin-stained sections were examined, after which selected slides were stained with the Giemsa technique.

Inclusion bodies were also studied by means of rat-embryo tissue culture (*1*). These were fixed in methyl alcohol and stained by the Giemsa method when cytopathic effects resulting from infection with RV had become pronounced.

RESULTS

Living Cultures in Gelatin Sponge-Matrix

Because of the optical properties of gelatin sponge it is not possible to observe in detail the cellular changes within the sponges in living cultures. In previous work with polyoma virus in this system, however, macrophages and fibroblasts on the glass adjacent to the sponges were much less numerous in infected than in control cultures after the 1st week of culture (*2*). This same phenomenon was observed in the cultures of rat salivary gland infected with RV. After 7 to 10 days of culture, large numbers of macrophages and small groups of fibroblasts migrated out from the sponges in the control cultures and eventually covered most of the flat portion of the tubes. In the infected cultures, only very small numbers of macrophages appeared on the glass, and even these were granular and appeared to be in poor condition. Also, as in the polyoma virus-mouse salivary gland system, the decrease of *p*H between fluid changes was less marked in the infected cultures than in the uninfected ones after the 10th day of culture. Presumably this sign of less extensive metabolic activity was a reflection of the smaller populations of macrophages and fibroblasts in the infected cultures.

Fluids removed from the salivary-gland cultures in the first set gave HA titers of 1:32, 1:64, and 1:64 on the 14th, 29th, and 32d days after exposure of the tissue to RV, while no hemagglutinins were demonstrated in fluids from the control cultures, taken at the same intervals. With the dilution effect of fluid exchanges considered, it would appear that hemagglutinins were produced by the infected cultures.

Fixed Sponge-Matrix Cultures

A response to RV was observed at all time intervals studied. Intranuclear inclusion bodies were found, both in epithelial and in connective-tissue cells (figs. 1 and 2), at the earliest (10-day) interval as well as at the latest (32-day) interval after infection. These inclusion bodies were not seen in any uninfected control cultures. They tended to be more

numerous, larger, and more eosinophilic than those previously observed in mouse salivary gland infected with polyoma virus, but were nontheless very similar. Usually the inclusions were surrounded by nonstaining zones, and the nuclear chromatin was distinctly marginated or beaded (figs. 1, 2, and 3). Often it was possible to identify a nucleolus near the inclusion body. Occasionally more than one inclusion body was seen in a single nucleus. Irregularly outlined inclusions were sometimes seen, just as in polyoma virus-infected cultures of mouse tissue, but most often they displayed a smooth, round, or oval contour. Disintegrating cells were noted in which it was still possible to discern the inclusions. In these, the inclusions usually occupied nearly all the nuclear volume and were weakly eosinophilic with a homogeneous, hyalin appearance. The cytoplasm of such cells was either fragmented or no longer present.

Other nuclear alterations, similar to those previously observed in polyoma virus-infected cultures of mouse tissues, were noted. One was the occurrence of approximately circular, clear areas within the nuclei ("ballooned" nuclei, *see* fig. 3). Inside these clear areas were very small, weakly eosinophilic granules, rather loosely dispersed. Still other nuclei showed multiple, irregular coarse fragments of eosinophilic material arranged in a rosette about the nucleolus. These irregular eosinophilic bodies appeared to represent variants of the more typical single inclusion bodies. It was our impression that single, large inclusion bodies might sometimes be derived from a fusion of the smaller eosinophilic bodies.

With the Giemsa technique, the inclusion bodies gave an intense deep-purple reaction characteristic of Type-A inclusion bodies. The smaller, irregular intranuclear bodies found in rosettes around nucleoli gave a similar reaction. No structures in addition to those seen in hematoxylin-eosin preparations were revealed by the Giemsa stain.

Differences were noted in the organizational pattern of the cells in the infected cultures as compared with the uninfected cultures. Although necrosis was present in both the infected and the control cultures at any given time interval, the connective-tissue elements were more selectively and more completely reduced in the infected cultures (figs. 4 and 5).

Secondary to this destruction of connective tissue, there was a tendency toward rearrangement of the viable epithelial cells in the infected cultures. Thus, as illustrated in figure 4, the acinar pattern in portions of the explants was lost, and the remaining cells formed narrow strands, sometimes extending out of the explant and a short distance into the adjacent gelatin sponge matrix. In contrast (fig. 5) the integrity of the ductal and acinar structures was maintained in the uninfected cultures, even though the epithelial cells showed atrophic changes and loss of morphologic signs of functional activity. This maintenance of organotypic structure appeared related to the preservation of the connective tissues in the uninfected cultures.

This "regrouping" and migration phenomenon was observed in 10-day, as well as in older, cultures (figs. 6 and 7). It was not associated with proliferative activity, and for this reason the cultures never duplicated

the picture of active growth so characteristic of cultures of mouse salivary gland infected with polyoma virus. However, there was evidence of a small amount of mitotic activity in rat tissue infected with RV *in vitro*. Figure 8 illustrates a cluster of regrouped epithelial cells, situated in a mass of necrotic tissue. These cells have large nuclei and basophilic cytoplasm, and among them is one cell containing an intranuclear inclusion body and another in the anaphase of mitosis. Thus, the response to RV included a mixture of cytolysis and cell division, but the latter was quantitatively insufficient to compensate for cell loss by lysis.

Monolayer Cultures of Rat Embryo

Giemsa-stained coverslip preparations of rat-embryo monolayer cultures were examined several days after infection with RV, before the cytopathic effect had become complete. In these preparations, the majority of the viable cells showed no changes, but scattered areas contained many cells of fibroblast-like morphology with intranuclear inclusions (figs. 9 and 10). The inclusion bodies appeared to be in various stages of development. The first detectable nuclear alteration was loss of distinctness of the delicate leptochromatic pattern of the chromatin. In successive stages, the sharp lines of the chromatin network gave way to broader, blurred bands in which the reticular pattern became indistinct. Condensation in the central part of the nucleus then took place, which produced a dark purple central mass surrounded by a violet-colored zone (fig. 9). At this stage the nuclear membrane was much more coarse and somewhat beaded. In the final stage, the central condensed area enlarged to fill all the nuclear volume except for a narrow clear zone, or halo, between it and the heavily marginated chromatin lining the nuclear membrane (fig. 10). Bits of deep purple-staining material similar to the inclusion bodies were found scattered around outside of cells and appeared to represent the remains of inclusions released from broken nuclei.

In these cultures there was no evidence of proliferative activity. The cell population of infected cultures was markedly smaller than that of the uninfected control cultures, which retained the character of a virtually uninterrupted monolayer.

DISCUSSION

The similarity of the intranuclear inclusions in rat tissue cultures infected with RV to the inclusions in mouse salivary-gland cultures infected with polyoma virus raises a question as to possible contamination of the rat cultures with polyoma virus. This possibility is excluded by the fact that previous studies (*5*) on cultures of rat salivary gland infected with polyoma virus failed to reveal development of intranuclear inclusions or any other changes described here with RV. Unpublished studies (*6*) of polyoma virus-infected cultures of rat submandibular gland rudiments

from 14- and 15-day embryos have also failed to show cytologic changes resembling those seen with RV.

Other extraneous viruses requiring consideration as possible contaminants responsible for the inclusion are the salivary-gland viruses (SGV), adenoviruses, and respiratory enteric orphan (REO) viruses. Species-specific salivary-gland viruses causing cytomegalic inclusion disease are well known in mice, guinea pigs, and man, and probably an analogous virus is responsible for a similar disease reported in wild rats (*7*). The size of the inclusions and the nuclear gigantism in cultured cells infected with the mouse salivary-gland virus (*8*) may be taken to distinguish the effects of SGV and RV, but Andrewes observed (*9*) that the inclusion bodies in cultures infected with guinea-pig SGV were never as large as those seen *in vivo*. Grand (*10*) found that heat treatment at 56 to 60° C for 30 minutes inactivated the ability of mouse SGV to produce inclusion bodies *in vitro*. Heating at 65° C for 15 minutes in our second culture series did not prevent development of inclusion bodies.

Adenoviruses also produce Type-A intranuclear inclusion bodies. Viruses of this group, however, are generally heat-sensitive and they too are presumed to have been eliminated, if originally present, from the second set of cultures by the preliminary heat treatment of the inoculum.

Inclusions associated with REO virus infections are typically intracytoplasmic (*11*) and morphologically unlike those found with RV in this study. HI tests, kindly performed in the laboratory of Dr. W. P. Rowe, National Institutes of Health, with normal and RV-immune guinea-pig serums, gave no indication of serologic cross-reactions between RV and REO viruses Types I, II, and III.

As mentioned earlier, the pathogenic potentialities of RV upon injection in large doses into nonimmune newborn rats are unknown because of the universality of natural infection with this agent in rat colonies. By analogy with the polyoma-virus infection in mice, the offspring of immune mothers would be expected to be refractory to tumor induction by RV. Inclusion bodies of the type described here in tissue cultures have not been seen in rats naturally infected with RV. However, it was recently reported (*12, 13*) that RV causes acute fatal infections in suckling hamsters. Histologic observations on these animals have shown a number of lesions. Among these were patchy congestion of alveolar capillaries in the lungs associated with focal intra-alveolar transudate and hemorrhages, subcapsular hemorrhages in the kidneys, severe congestion of vessels in the mucosa and serosa of the bowel, sometimes accompanied by gross intraluminal hemorrhages, and abnormalities of bone and tooth formation. These lesions are similar to those described by Toolan (*14*) and Dalldorf (*15*) in hamster "mongolism" after the injection of filtrates of human tumors transplanted to rats. The gross appearance of hamsters infected with RV (*12*) but surviving to weaning age is similar to that of the "mongoloid" dwarfs described by Toolan (*14*).

In spite of the many similarities between polyoma virus and RV, there remain some outstanding differences between these two agents. One is

the difference(s) in antigenic properties, with no evidence (in the form of cross-reactions) of antigenic determinants common to the two. Another is the apparent, but not fully established, lack of oncogenic activity in RV, as compared with the known highly oncogenic activity of certain strains of polyoma virus. One can only speculate as to whether these two differences are interrelated and mutually dependent on differences in genetic constitution of the two viruses. It would be instructive, however, to make a comparative study of the pathologic reactions, exclusive of tumor development, to the two viruses *in vivo*. It seems plausible that two agents with so many physical and biologic properties in common might project their similarities in some degree to the disease patterns causally related to them. The appearance of morphologically similar inclusion bodies in response to both polyoma virus and RV may be taken as evidence that this is true, but it must be kept in mind that Type-A inclusion bodies are produced by viruses of widely differing pathogenic activities. If, on the other hand, antigenic characteristics are the main determinants of patterns of susceptibility and response in host animals, then one might expect to find very little in common in the disease processes produced by the two viruses.

To accept *a priori* the validity of the organotypic culture system as a means of detecting viral oncogenic activity, the results obtained here must be interpreted to favor a prediction that RV of the samples used would not show oncogenic activity on the rat salivary gland *in vivo* in a degree comparable to that demonstrated for certain strains of polyoma virus in the mouse. The observation of a small degree of mitotic activity in the infected cultures suggests, however, that some small potential for induction of rat salivary-gland tumors exists in this virus. It is conceivable that more active variants of RV might be selected or created by natural or laboratory processes, or that RV might prove to be oncogenic in other tissues or species.

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PLATE 27

FIGURE 1.—Acinus in 18-day-old culture of rat submandibular gland infected with RV. *Arrows* indicate two epithelial cells with nuclei containing inclusion bodies. Marginated chromatin is clearly visible in nucleus on *right*. Nucleus on *left* contains two inclusion bodies abutting on one another. Hematoxylin-eosin. × 1,340

FIGURE 2.—Another field from culture shown in figure 1. Cell in connective tissue (*arrow*) contains intranuclear inclusion body. Epithelial cells to *right* of inclusion body have lost glandular pattern. Cell in *lower center* is undergoing disintegration. Hematoxylin-eosin. × 1,340

FIGURE 3.—Group of epithelial cells from same culture shown in figures 1 and 2. One nucleus shows ballooning phenomenon, another contains intranuclear inclusion body. Small eosinophilic particles are barely visible within ballooned nucleus. Hematoxylin-eosin. × 1,340

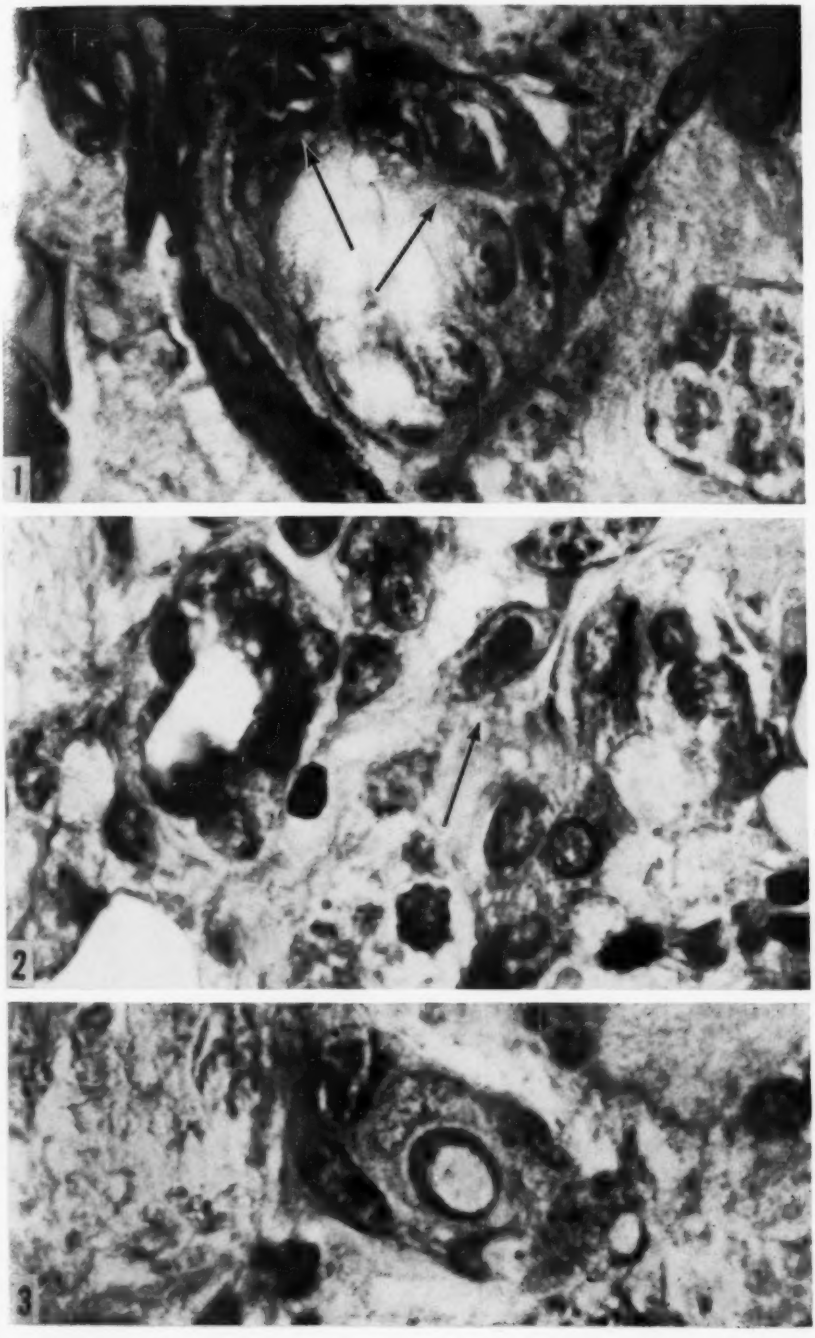

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PLATE 28

FIGURE 4.—RV-infected culture of rat submandibular gland after 32 days in culture. *Note* partial disarrangement of organoid pattern, loss of connective-tissue cells, and extension of groups of epithelial cells into gelatin sponge adjacent to explant. Hematoxylin-eosin. × 260

FIGURE 5.—Uninfected control culture of rat submandibular gland after 32 days in culture. Organoid structure is largely intact and connective-tissue cells survive within collagenous groundwork. Acini are atrophic, but intact. Hematoxylin-eosin. × 260

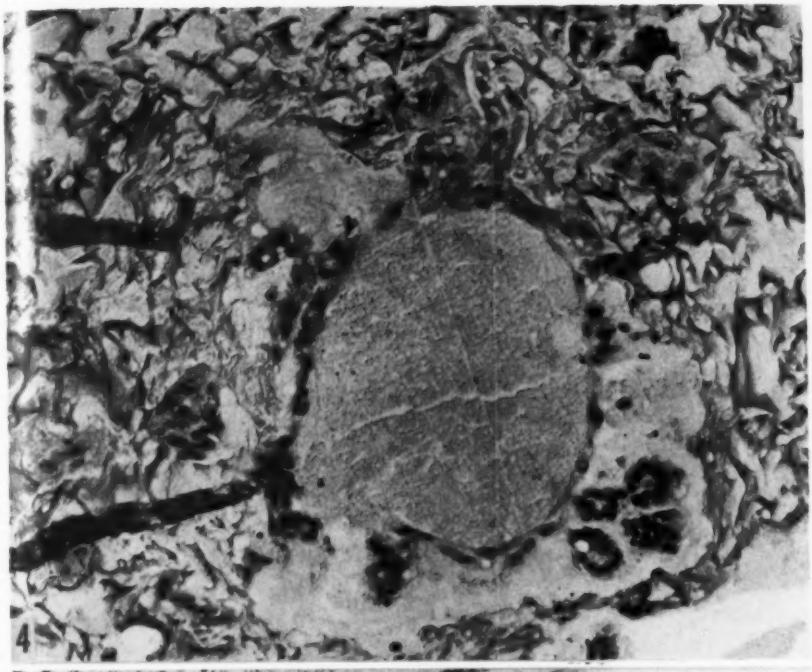
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PLATE 29

FIGURE 6.—RV-infected rat submandibular gland after 10 days in culture. Necrosis of connective-tissue cells is incomplete. At *lower right* epithelial cells form clusters occupying adjacent compartments of gelatin sponge. Hematoxylin-eosin. × 260

FIGURE 7.—Higher magnification of portion of area at *lower right* in figure 6. Although these cells appear to be of epithelial origin, orientation in glandular pattern is lacking. Cell in *upper center* contains intranuclear inclusion body. Hematoxyiin-eosin. × 1,340

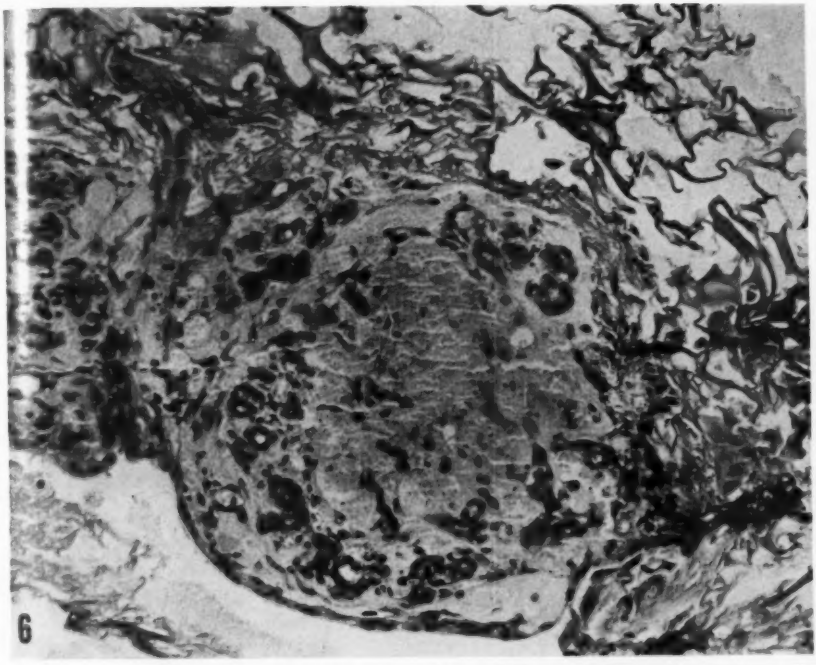

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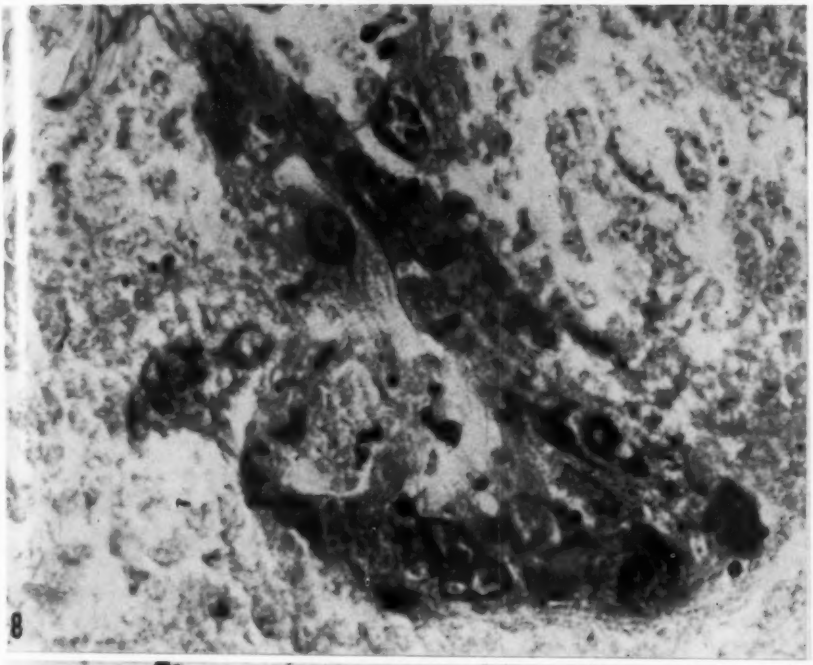
PLATE 30

FIGURE 8.—RV-infected rat submandibular gland after 18 days in culture. This disarranged group of epithelial cells lies in a bed of completely necrotic tissue. Several cells in this field are undergoing disintegration. At the same time, one cell is in anaphase of mitosis (*upper center*). Two cells at *lower center* contain intranuclear inclusion bodies. Hematoxylin-eosin. × 1,000

FIGURE 9.—RV-infected coverslip culture of trypsinized rat embryo. Intranuclear inclusion body at intermediate stage of development is shown. Chromatin is marginated and central densely stained area is apparent. Giemsa. × 1,000

FIGURE 10.—Another intranuclear inclusion in same culture shown in figure 9, but at late stage of development. Margination of chromatin is more pronounced and densely stained area fills nucleus except for narrow pale zone between the inclusion and nuclear membrane. Giemsa. × 1,000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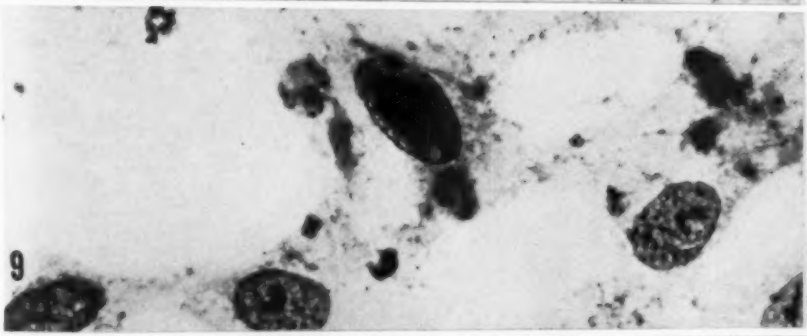
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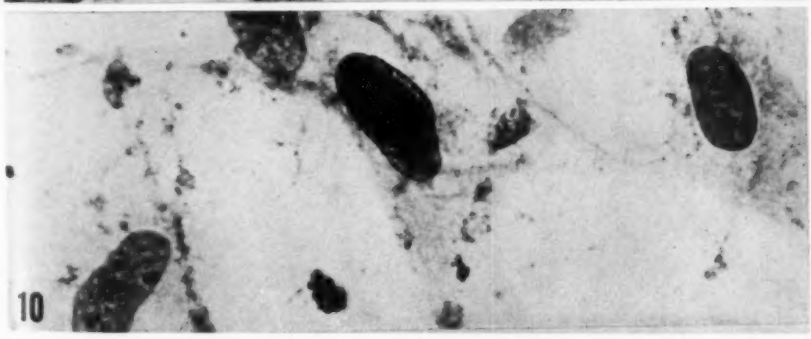
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Announcements

Eleanor Roosevelt International Cancer Fellowships

The International Union Against Cancer, through funds made available by the Eleanor Roosevelt Cancer Foundation, will award annually fellowships for research on cancer. These are senior postdoctoral awards designed to support investigators who have demonstrated interest and outstanding ability in the field of basic research on cancer, its experimental and clinical aspects, and who wish to broaden their experience by study in another country.

The duration of the fellowships ordinarily will be 1 year, but this period may be extended or shortened in special circumstances. The stipend will be based on the current salary of the applicant and the salary of persons of comparable qualifications in the place where the applicant expects to study. An allowance will be made for dependents and costs of travel.

Application forms and additional information may be obtained from The International Union Against Cancer, P.O. Box 400, Geneva 2, Switzerland.

Erratum

Volume 26, Number 5, May, 1961

Page 1071, under "Discussion," last line, in the paper entitled "Enzymatic Deamination of Deoxyadenylic and Adenylic Acids by Normal and Cancerous Rat Liver Tissues," by Fiala and Kasinsky, *for* "Unlike the increased activity of dAMP deaminase, however, the increased level of A deaminase is not an essential feature of tumors," *read* "Unlike the increased activity of dCMP (deoxycytidylic acid) deaminase. . . ."

Manuscripts Approved for JNCI, May 15 to June 15, 1961

"Intrasplenic transplantation of neonatal thymus," by Roger W. O'Gara and Jewel Ards.

"Incidence of leukemia and other tumors in thymectomized irradiated mice bearing thymic transplants," by Roger W. O'Gara and Jewel Ards.

"Lysis of homologous cells by sensitized lymphocytes in tissue culture," by Werner Rosenau and Henry D. Moon.

"Mammalian chromosomes *in vitro*. XV. Patterns of transformation," by T. C. Hsu, Daniel Billen, and Albert Levan.

"Glucose metabolism of Rous sarcoma virus-induced tumors. I. Comparison of chorio-allantoic membrane (CAM) and Rous sarcoma virus-induced tumor of the chorio-allantosis (RSV-tumor)" by Alvin S. Levine, Richard Uhl, and James Ashmore.
"Mode of inheritance of resistance to Rous sarcoma virus in chickens," by Nelson F. Waters and B. R. Burmester.

"The cytotoxicity of a horse anti-human cancer serum for normal and malignant cells *in vitro*," by Milton N. Goldstein and Raymond Hiramoto.

"Production of hyaluronic acid by fibroblasts growing from explants of Walker tumor 256. Production of hyaluronidase by the tumor cells," by Henry Grossfeld.

"Carcinogenesis of the uterine cervix in mice: Effect of diethylstilbestrol following limited application of 3-methylcholanthrene," by Edwin D. Murphy.

"The hepatocarcinogenicity of a series of 4'-alkyl-4-dimethylaminoazobenzenes," by Ellis V. Brown and Ali A. Hamdan.

"The β-glucuronidase activities of cultured cells derived from C3H mouse liver," by Edward L. Kuff and Virginia J. Evans.

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